

How not to compete with tigers

Australia should take a lesson from its Asia-Pacific neighbours before it undermines its research and higher education infrastructure by slashing budgets for its universities and cutting incentives for industrial research.

ONLY last year, the previous Australian government was emphasizing how important it is for that country to develop research links with the rapidly developing economies of Asia. It is ironic, therefore, that the new government's approach to science and higher education (see page 659) could not stand in more unfavourable contrast to others in the Asia-Pacific region.

The 5 per cent cuts in operating funds for universities and the raising of fees for students in science and medicine (see *Nature* 382, 569; 1996) may not be as bad as had been anticipated a few months ago, but that is small comfort. There is a worldwide trend among young people to turn their backs on careers in research, and what better way to ensure that that trend gets worse in Australia than to impose massive increases in fees for students in science, medicine, engineering and veterinary science while holding those for arts and social sciences at a much lower level?

The cuts and fee increases are being introduced as part of a package of measures to slash more than A\$7 billion from government spending to try to eliminate a budget deficit without raising taxes. But recent polls show that a majority of Australians oppose squeezing money out of higher education. Instead, they might even accept increases in their comparatively low taxes.

Singapore provides one example of the wholly different approach adopted by Australia's neighbours. Ten years ago, the Singapore government's investment in research and development was negligible. But realizing that it can no longer rely on low-end manufacture of products using cheap labour or trade to survive, the Singapore government has done a remarkable job of pumping money into its universities and new institutes associated with them.

Singapore is not deliberately aiming to support 'blue-sky' research. Rather, it is looking for practical returns for its industry, which it is actively and effectively encouraging to invest in research and development. Nevertheless, its policy over the past five years, led by the National Science and Technology Board which has pumped an extra US\$2 billion into research, has had a beneficial effect on basic research and science education in Singapore.

The Singapore government, sensitive to the growing trend among young people to turn their backs on science in favour of more financially lucrative careers, is going to great lengths to encourage school-children to pursue careers in research, with science competitions and scholarships to entice the brightest into science and engineering departments in universities. It is also working hard to ensure that there will be research-related jobs for them in local industry when they graduate, by providing incentives to industry to build a research infrastructure. Such efforts make the Australian government's plans to increase fees for science courses while cutting tax incentives for industrial research look short-sighted at best.

The Australian Council of Deans of Education has predicted a serious shortfall of primary and secondary school teachers early next decade because of state education cuts that have already been made. The latest moves seem certain to reduce enrolment in higher-cost science and technology courses at universities, making the teacher shortage even worse in science and mathematics, the very areas that need to be promoted.

The Australian government may respond that it has to grapple with a very different economy from that of Singapore (and other

booming economies in Asia). Rapid growth and the high savings rates in Asia make it easier to place strong investments in the universities and research and development infrastructure. Australia, on the other hand, has to struggle with slow growth and a declining savings rate. But, as its neighbours seem to have grasped, weakening the science and technology base will undermine the economy in the longer run. □

It's the platform, stupid

The Republican party in the United States has made the Democrats look strong in science policy.

THE 'party platform' endorsed by Republicans in San Diego, California, last week will have no binding hold on Bob Dole, the party's presidential candidate, who helpfully says that he has not bothered to read it. But the document offers the only available guide to what a Republican Congress and a Dole presidency would actually do after November, given the chance.

The platform's comments on science and technology kick off with an implicit endorsement: "A recent report by the Office of Technology Assessment [OTA] attributes at least half of all economic growth in the United States to advances in technology." What a useful OTA that was before the Republican Congress abolished it last year, in the face of objections from scientists and engineers of every political hue.

The Republicans go on to reaffirm their support for health research and the National Institutes of Health. They say that federal programmes "must emphasise basic research" and call for "private sector funding of applied research" encouraged by unspecified changes in taxation and regulation. But reliance on private-sector funding apparently need not extend to the Department of Defense (DoD) or the National Aeronautics and Space Administration — which between them spend four-fifths of government funds for applied research and development. The platform document accuses Clinton of "decimating" military research and development and "denying crucial resources" to the space programme. The Republicans repeat their long-standing pledge to eliminate the energy department, with the nuclear weapons laboratories going to DoD. The rest of the department would be "farmed out to other departments and offices". An inspiring vision indeed for Fermilab and Brookhaven.

These proposals are a rehash of the two-year-old Contract with America. They indicate that the Republicans have learned very little from their failures since then, and reflect a dearth of serious thinking about science and technology issues on the American right. Furthermore, the Republicans lack staff expertise to match that of their long-entrenched Democrat opponents, and rely too much on advice from industry-funded pseudo- 'think tanks', such as the Heritage Foundation.

The Clinton administration's Office of Science and Technology Policy has been ineffectual at times, but at least it has had some idea of what it is trying to do. This vapid little document suggests that the Republicans have, as yet, no idea at all. □



Ethics worries over execution twist to Internet's 'visible man'

Washington. The 'visible man' is known to thousands of Internet users. An extremely detailed three-dimensional atlas of the male human body, it was placed on the Internet two years ago, launching the US\$1.4 million Visible Human Project of the US National Institutes of Health (NIH)'s National Library of Medicine (NLM). What fewer people know is that the digital images were taken from the cadaver of an executed Texas prisoner, Joseph Paul Jernigan.

Magnetic resonance images, radiographs and computerized tomography (CT) scans were made of his body, before it was frozen, sliced into 1,800 1-mm sections and photographed again.

The value of the anatomical database is undisputed. But specialists in medical ethics and lawyers working with inmates on death row say the use of an executed man raises disturbing questions.

Jernigan was aged 39 in 1993 when he was executed by lethal injection in Huntsville, Texas, for killing an elderly man, Edward Hale, during a burglary in 1981. Jernigan donated his body to science. His signed consent left his body to the Anatomical Board of the State of Texas.

Vic Spitzer, an anatomist who is one of the two principal investigators on the project at the University of Colorado Medical Sciences Center, says that the use of an executed man was a boon, in that the body was fresh, and allowed the best possible magnetic resonance images and CT scans.

'Still warm' might be a more accurate description. The anatomical board arranged to pick up the body immediately after the execution. They took it to a funeral home in Huntsville for early preparations and then put it on a charter jet to Denver where it was met by the Colorado researchers. About eight hours after the execution, they began taking images of the corpse.

"It was extremely convenient," says Michael Ackerman, the NLM official overseeing the project. Spitzer adds: "It's very difficult to find an intact, non-traumatized, non-pathological cadaver. I'm not condoning execution. But I don't believe in wasting resources either."

Andrew F. Payer, secretary of the anatomical board, insists that the use of an executed man raises no ethical dilemma. The process "was in the letter of the law of the state of Texas. There was good intent both on Mr Jernigan's [side] and ours."

Payer says some reservations were expressed by NLM staff who feared a "public relations nightmare". He advised NLM

that it should follow the standard procedure of releasing the cause of death of the donated body — "a drug overdose" — but not Jernigan's identity.

Although Jernigan donated his body to science, the issues raised by its use in the on-line anatomical kit are many and contentious. Ethics specialists and prisoners' advocates question whether a prisoner on death row can be considered to be in a fit

IMAGE
UNAMIGABLE
FOR A COMBAT
FOR REASON
REASONS

An important resource: Jernigan's feet immortalized.

state to give free and informed consent.

George Kendall, a staff attorney with the Legal Defense Fund, a civil rights law firm, says: "A decision made under those circumstances would hardly be voluntary. When you're ready to be executed, you're hardly in the frame of mind to make some sort of long-term judgement."

Kendall is also concerned that donations could be "very easily abused" by prison officials and medical researchers. Arthur Caplan, the director of the Center for Bioethics at the University of Pennsylvania, agrees: "The question is, is it fair to use someone in that setting for research?"

Caplan argues that the possibility of coercion is "always there" when dealing with prisoners whose "liberty and choice is limited by being on death row".

Others question the validity of Jernigan's consent on the grounds that he was unaware of how his body would be used. Marcel LaFollette, a research professor at George Washington University in Washington DC who specializes in ethics and science policy, asks: "Did the consent include knowledge that he's going to be uploaded on the Internet and downloaded into everyone's personal computer?"

Offering inmates on death row the option of donating their bodies to science might encourage them to give up their efforts to appeal, and to "sign their own death warrants," claims Palmer Singleton, a death row

lawyer at the Southern Center for Human Rights, a group that argues against capital punishment. "The more outs that you give them for bringing a quick end to what has been a terrible ordeal, the more you run the risk of covering up substantial profound wrongs."

Some ethics specialists consider that the use of Jernigan's body was ethical, provided he gave his consent voluntarily. "I don't believe [free and] informed consent is meaningless on death row," says David DeGrazia, a biomedical ethicist at George Washington University. Similarly, Spitzer describes as "absurd" complaints about the legitimacy of consent in the case. "It's a phenomenal gift of people that donate their bodies to science and teaching. I hate to see that gift distorted by a process of someone that happens to be on death row."

Nonetheless, Spitzer says that his group "consumed a lot of hours of worrying and discussion" about the ethics of the situation. They turned to NLM, where senior staff "couldn't find any ethical or legal problems" according to Ackerman.

Later, however, NLM "had a real problem with it", says Spitzer. Concerned that Jernigan's family might sue, the library asked the Texas board to seek its approval. Payer refused. "There was no reason to go back to the family," he says. "Everything was done up front, and all according to the rules."

Mark Ticer, a lawyer who represented Jernigan, also dismisses the criticism. When Jernigan told Ticer of his intention to donate his body to science "a month or so" before the execution, he did not "feel like somebody was with a gun to his head forcing him to do it", says Ticer. The donation was "the only way that he felt like he could give something back for what he had taken". Wilmer Hale, a nephew of the man Jernigan murdered, also applauds the donation as "a good thing".

Jernigan's legacy to science is being seen by people around the world. The 55 gigabytes of full-colour 'Visible Human' images can be downloaded free from the Internet, provided users obtain a licence from NLM and credit it for any use made of the material. Around 600 individuals, corporations, high schools, universities and medical schools in 26 countries now have licences.

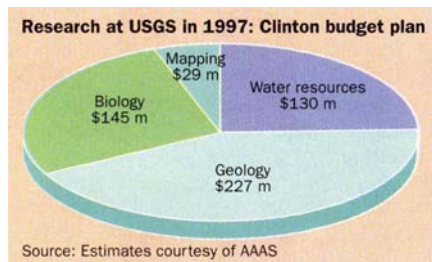
Jernigan has now been joined in cyberspace by the 'Visible Woman', an anonymous 59-year-old Maryland woman who died of a heart attack. **Meredith Wadman**

US Geological Survey picks up the NBS pieces

Providence, Rhode Island. A bold experiment to engage US government scientists in the systematic study of ecosystems will end quietly next month, when the two-year-old National Biological Service (NBS) is abolished and its operations transferred to the US Geological Survey (USGS).

Sixteen hundred staff at NBS's 40 field stations will become the Biological Resources Division of the USGS from October. Many of them are optimistic that they will be able to work more closely with hydrologists, geologists and map-makers in the USGS. But the change marks the defeat of the contentious plan of Bruce Babbitt, the interior secretary, to establish a powerful new agency dedicated to ecology (see *Nature* 365, 479; 1993 & 367, 400; 1994).

Ron Pulliam, the director of the NBS, intends to return to the University of Georgia, having declined to lead the new USGS division. He joined NBS in Washington two years ago to lead an extensive new project,



but has spent his time locked in a battle with Congress for his agency's survival. But he told NBS staff at the annual meeting of the Ecological Society of America in Providence, Rhode Island, that the USGS was "a good, safe place" for them to end up.

The NBS was founded as the National Biological Survey in 1994 from the science operations of three agencies of the Department of the Interior — the Fish and Wildlife Service, the Bureau of Land Management and the National Park Service. The plan was to expand these operations to form an agency that would focus on ecology and biodiversity.

But the plan quickly ran into serious political trouble. Even the Democrat-controlled Congress of 1993–94 sided with landowners who feared the impact of the NBS on land use. In a doomed bid to assuage this fear, the agency was renamed the National Biological Service.

The Republican Congress elected in November 1994 moved quickly to abolish the NBS, but it did not want to close the field stations (these are cleverly scattered across 38 states) and instead found a home for them in the USGS.

Gordon Eaton, director of USGS — which was itself threatened with abolition at the outset of this Congress, until the new majority discovered what the agency actually does — says he is "quite bullish on this marriage". He says he is "very excited about all the opportunities" that will arise for collaboration with the new division.

But Eaton acknowledges that morale among the staff has hit rock bottom. "These people have been through hell," he says, noting that they were uprooted from their old agencies, "promised a grand new future", and are now being uprooted again. "But they are beginning to see that they'll get more respect here than they did in the agencies they originated from," he says.

Within the enlarged USGS, the Water Resources Division will remain the dominant division, with 5,000 staff. Geology, mapping and the new biology division each have around one-third as many people. In terms of research, however, the new division will be the second largest in USGS, accounting for about 30 per cent of all research there (see pie chart).

The enlarged USGS, embracing more than 90 per cent of all research at the Department of the Interior, may be the shape of things to come in the federal government, says Eaton. "There will be fewer, larger units, and they will be multi-disciplinary," he predicts.

Some biologists joining the agency are worried about USGS publication rules, which they say will make it hard to present new science without going through months of internal review. But agency officials say that the new division will retain autonomy in this and other administrative matters. On the whole, NBS staff at the Ecological Society meeting were enthusiastic about the change, and the opportunities it brings, especially in mapping and hydrology.

The change may bring some stability to their lives. But their identity crisis could resume soon: Eaton and others think that the USGS's name should eventually change, to something like the National Natural Resources Agency, to reflect the agency's interests beyond rocks. Babbitt is against the change, however, understandably fearing that it would risk attracting more unwanted attention from the Congress.

Colin Macilwain



Gordon Eaton: time to think big.

US superconductor research is good value — so far

Washington. A \$50 million US programme to develop power applications for superconductors has chalked up impressive successes, despite being only half the size of a comparable programme in Japan, says a US panel that compared the two. The US Department of Energy programme has achieved "very good coupling of government and industry funding, and of applications people and fundamental research people," says David Larbalestier of the materials science department at the University of Wisconsin at Madison, who chaired the panel.

But the Japanese programme benefits from stable funding and "a strong national belief that superconductivity will be vital 21st century technology" according to a preliminary summary of the study, conducted for the World Technology Evaluation Center (WTEC), a government-funded group based at Loyola College in Baltimore, Maryland.

Japan will ultimately benefit from its approach of seeking applications for established, metal low-temperature superconductors (LTS) as well as for the more exotic, ceramic high-temperature superconductors (HTS), the panel reports. Recent advances enabling LTS materials to be supercooled by a refrigerator, removing the need for a flow of liquid helium, will increase the practical value of these materials, Larbalestier says.

The panel also reviewed the German programme, the third largest national effort. This has improved over the past four years, but according to Larbalestier is "not leading in any major area". Germany's spending is close to that of the United States at \$40 million annually. Japan spends \$100 million annually on power applications of superconductors, and the work also benefits from Japan's huge effort to develop new Maglev (magnetic levitation) trains.

Superconductor application research was rescued from near obscurity in 1986 by the discovery of a new class of ceramics that could conduct electricity without resistance at far higher temperatures than the established, metal-alloy superconductors. "The tenth anniversary is coming up," says Larbalestier. "A lot of promises were made that really had no hope of being put into action." Progress in perfecting the new materials has been steady but unspectacular.

US successes noted by the panel include a 150-kW electric motor and a 50-m-long power cable, each using HTS and each developed in a partnership between Department of Energy laboratories, universities and the electricity supply industry.

But the panel is worried that funding — from the politically contentious renewable energy division of the department — "has to be fought for each year".

C. M.

Budget cuts hit Australian industry R&D

Canberra. In its first budget since coming to power in March, the new conservative Coalition government of the prime minister, John Howard, has reduced from A\$790 million to A\$341 million annual funding for a system of tax breaks designed to encourage research. But major public research programmes emerged relatively unscathed from the austerity budget, aimed at reducing the country's budget deficit by A\$4 billion over the next year.

The cut follows the government's closure last month of another scheme to promote industrial research, a "syndication" scheme, which allowed pooling of investments in research and development (R&D) to attract tax concessions. This saved the government some A\$225 million annually. Similarly the government last week announced a 5 per cent cut in funding for the universities (see *Nature* 382, 569; 1996).

The treasurer, Peter Costello, is relying on an improvement in business confidence and investment to spur growth in the economy. The effect of the cuts on support for industrial research will be watched closely. The former Labor government has introduced the tax breaks schemes as an incen-

tive for Australia to become more innovative and competitive. Over the decade that the programme has been in operation company spending on research has increased considerably from a low starting point.

The effects of the cuts may be offset to some extent by a new programme (the "Start Program"), announced by John Moore, the industry and science minister, which will provide competitive grants, loans and subsidized interest rates. The scheme is estimated to cost A\$520 million over four years, about half the cost of the axed syndication scheme.

The national research agency, CSIRO, gets an extra A\$20 million over three years on an annual appropriation of A\$449.7 million. The innovative scheme of Cooperative Research Centres will be maintained with an A\$19-million increase in funding to A\$150 million for 64 centres, one of which will focus on space research. The Australian Research Council, which makes competitive grants to academics under the education ministry of Amanda Vanstone, will receive A\$396.2 million (up from A\$346.4 million), with most of the increase going towards infrastructure for university research.

While changes to public and private health insurance dominated the statements by Michael Wooldridge, the health minister, medical research has been increased to A\$181.5 million. The government also offered "to match the offer of funding from the Wellcome Foundation in Great Britain which will mean significant additional funding for biomedical research in Australia". No figures are yet available.

In its Innovation Statement last December the former Labor government unveiled a package of measures to improve Australia's capabilities in basic research and apply it to economic goals (see *Nature* 378, 653; 1995). Seven Major National Research Facilities approved then have survived unscathed with Labor's grant of \$62.4 million, but the new government has saved A\$20 million by axing two of Labor's "Innovation Flagships", allowing only an institute for magnetic resonance diagnosis of cancer to proceed for A\$3 million.

Speaking of the impact of the budget on science, Peter McGauran, minister of science and technology, said: "Our commitment will maintain Australia's world class science and research effort". Peter Pockley

Threat of ban hangs over Israeli archaeology

Jerusalem. Israeli archaeologists were dismayed this month when Meir Porush, deputy minister of housing and construction, ordered the Israel Antiquities Authority to abandon a salvage dig because the site may contain ancient Jewish graves. They fear that the decision, the latest in a series of restrictions on archaeological work, may make it next to impossible to document and preserve Israel's ancient history.

The decision also raises the issue of where the new right-wing Likud government will stand in the long-running conflict between archaeologists and the ultra-orthodox groups who contend that excavation of graves violates Jewish religious law.

The groups made an important gain two years ago when the attorney-general, Michael Ben-Yair, ruled that under Israeli law human remains are not "antiquities". This means that researchers are not allowed to study bones they discover, but must hand them over to the Ministry of Religious Affairs for reburial.

Many archaeologists and anthropologists oppose the ruling, arguing that the study of human remains provides important historical and scientific information. They are concerned by the prospect that ultra-orthodox groups might succeed in having the ban on studying human remains extended to include work on the graves themselves. That would make it

almost impossible to excavate, they say, because ancient graves are strewn all over Israel.

The dig at the centre of the current dispute is at a major construction site in Modi'in, a new town being built halfway between Jerusalem and Tel Aviv. Under Israel's antiquities law, whenever a site is deemed of historic or archaeological interest, the contractors must call in the antiquities authority to carry out a salvage dig before work proceeds. If the site turns out to be of particular importance, the authority may ask for construction plans to be revised, but usually the work proceeds after the site has been documented.

Because human remains are often found at salvage digs, they have frequently become the scene of violent demonstrations. Atra Kadisha, the ultra-orthodox organization that champions the fight against the excavation of graves, claims that Jewish religious law forbids the opening of graves under almost any circumstances. It insists that all graves must be left undisturbed, and roads and building schemes redesigned to bypass them.

"Tampering with bones" causes the deceased person's soul to suffer, says Rabbi David Schmidl, a member of the board of Atra Kadisha. "Even if there is important science to be found in a grave, it

can't be violated," he says. But other orthodox groups say that Jewish law permits both the transfer of human remains to a new site and some non-invasive studies of bones before reburial.

The ban on the dig ordered by Porush — a member of the fundamentalist Agudat Yisrael party — has reinforced concern that ultra-orthodox Jews may try to impose their beliefs on the rest of the population. In Israel's May elections, the two ultra-orthodox parties won 14 out of 120 seats in parliament, the Knesset, and they now hold several important government positions. The National Religious Party, which takes a more moderate line on excavations, won nine seats.

During the horse-trading to form a coalition government that followed the elections, Benjamin Netanyahu, the prime minister, promised the three religious parties that joined his government that all disputes over the excavation of graves would be resolved by a government committee. The committee has not yet been set up, but religious parties are said to understand that it will be sympathetic to their demands. Yossi Levi, the antiquities authority's chief archaeologist for the central region of the country, says that if the planned building scheme is abandoned, the site would at least "remain accessible to archaeologists rather than being built over."

Haim Watzman

Roche faces charges over *Taq* patent claim

Munich. Swiss pharmaceuticals company Hoffmann-La Roche and US biotechnology company Cetus face charges of intent to mislead the US Patent and Trademark Office (PTO) in their claims to native *Taq* DNA polymerase, the key enzyme in the polymerase chain reaction (PCR).

A federal court ruled last week that Cetus — the company originally granted the patent in 1989 — acted with “gross negligence” in withholding relevant information from the PTO. The court has set a hearing for next month to discuss whether the company acted with intent to mislead, and to fix a date for a trial. If intent is proved, the patent would be declared unenforceable.

Hoffmann-La Roche bought the patent rights for native *Taq*, along with a bundle of related patents, including recombinant *Taq* polymerase and the PCR methodology itself, for US\$300 million in 1991. The long-running court case began four years ago when Roche sued Promega, a US laboratory supply company, for patent infringement. Promega had refused to sign a new licensing agreement that would have increased its

royalty payments fivefold.

Promega shifted the focus of the case by counterclaiming that the patent on native *Taq* polymerase should be declared invalid. It also called for Roche's other PCR-related patents to be declared invalid. The key issue in Promega's arguments is whether the enzyme extracted from the bacterium *Thermus aquaticus* described in the Cetus patent is the same as that extracted by scientists from the University of Cincinnati in 1976 and by a Russian group in 1980 (see *Nature* 373, 377; 1995).

Cetus argued that its enzyme was different, and therefore patentable. This issue is widely contested. Only last month the European Patent Office rejected a patent application from Roche for native *Taq* because it believed the enzymes to be the same (see *Nature* 381, 100; 1996).

Promega presented to the US court more than a dozen pieces of evidence to support its contention that Cetus had supported its patent claim with arguments that were inconsistent with well-known scientific principles, or that Cetus had withheld relevant

information contained in its own laboratory notebooks. The court upheld five of Promega's claims.

The court agreed, for example, that Cetus had misrepresented scientific principles by claiming that the enzyme was unique because its apparent molecular weight was significantly higher than estimates reported earlier in the literature. Cetus had argued that this difference could not be accounted for by differences in methodology.

Cetus had claimed that the method of estimation of molecular weight used by the Cincinnati group — gel filtration chromatography, a sizing technique — was reliable, so the 20 kDa difference in molecular weight observed reflected true differences in the enzyme proteins purified.

But internal Cetus documents showed that the company knew that the method yielded unreliable estimates of molecular weight and the court ruled that Cetus had withheld relevant information.

Cetus had further claimed that, even if the enzymes were the same, the protein of lower molecular weight described by the Cincinnati group could not be the full-length enzyme but a smaller proteolytic breakdown product. Yet laboratory notebooks showed that Cetus had carried out experiments with a proteolytic fragment of *Taq* of the appropriate molecular weight. The fragment did not bind to the chromatography column used by the Cincinnati scientists to purify the enzyme.

In its defence, Roche says that binding is notoriously dependent on experimental conditions, and that even small differences could have explained the failure of the fragment to bind. Nonetheless, the court said that the company should have supplied the information, as it would have helped the PTO to assess Cetus's original patent claim.

The court dismissed Roche's claim that its enzyme was much purer than those previously extracted and that this alone justified its patent, arguing that the company had exaggerated the purity of its preparation. Roche says the judge miscalculated the purity value.

Tom White, head of Roche's US West Coast research and development department, is confident that the company will successfully defend itself against all the other charges, and prove there was no intent to mislead.

Promega is equally confident. “There have been so many instances of obvious misrepresentation that they can't be considered a series of mistakes,” says Randall Dimond, chief technical officer at Promega. Even if Roche wins this case, he says, it is unlikely to win the next stage, which will be a trial to consider the validity of the patent, given the current ruling of gross misconduct.

Alison Abbott

Free speech a thing of the past?

London. British universities are considering charging journalists on national newspapers for interviews with academics. The suggestion comes from the Committee of Vice-Chancellors and Principals (CVCP) in retaliation for a plan by newspapers to levy a £0.02 royalty (US\$0.03) on every article photocopied in a university.

The CVCP's proposal has provoked a mixture of outrage and bewilderment among scientists and journalists. “I find the idea pretty horrifying,” says Esther Pocock, a spokeswoman for the Biotechnology and Biological Sciences Research Council. “Scientists have a duty to explain to the public what they are doing”, she says, adding that that is an obligation written into research grants.

Pocock says that the introduction of fees would also diminish the quality of science reporting, explaining that scientists “must be willing to give advice freely” if they want to be reported accurately. Scientists may also be loath to bite the hand that feeds them; they need the media as much as the media need them.

Nigel Hawkes, science editor of *The Times*, thinks the idea of fees is “crude, totally unenforceable and ill-advised”. He

is not losing sleep, however: “I don't think it'll ever see the light of day. I can't see any publication approaching any scientist who is going to charge.”

Steve Jones, professor of genetics at University College London and a regular

newspaper columnist who has also written and broadcast radio and television programmes, says the proposal is “obviously wrong”. “Unless I am mistaken, universities are supposed to be in the business of disseminating knowledge.”

Both universities and institutions such as the

Natural History Museum in London charge commercial clients for science advice. But enquiries from the public and journalists are “always free of charge”, says Gina Dobson, a spokeswoman for the museum.

Whether the universities seriously intend to put an end to this practice remains to be seen. Graham Zellick — who is vice-chancellor-elect of the University of London, and represents CVCP in discussions on copyright with the Newspaper Licensing Agency, a body representing most of Britain's national dailies — was away on holiday as *Nature* went to press.

Ehsan Masood



Europe opens high-tech stockmarket...

Oxford. Millionaire European scientists may become commonplace following the launch next month of a pan-European stock exchange for high-growth companies.

A similar exchange in the United States, NASDAQ, nurtured the development of three of America's leading high technology giants, Microsoft, Intel and the biopharmaceutical blockbuster Amgen. Europe hopes to repeat the US success.

The high-tech focused pan-European stock exchange has been dubbed EASDAQ — the European Association of Securities Dealers Automated Quotation system. Its opening is expected to have three major impacts on Europe's technology base.

First, it should slow the flight of high-tech companies to the United States. Second, it should make it easier for scientists to get seed funding for ideas they have for new companies. And third, scientists could find themselves being turned into millionaires if the ideas underpinning their companies capture the investment community's imagination.

The new exchange is looking to attract young fast-growing companies in areas such as advanced materials, biotechnology, environmental technology, Internet service providers, medical technology and software houses. A number of companies have been lined up for the opening day of the new

exchange, including the Belgian biotechnology diagnostics company Innogenetics.

European financiers are offering high-growth companies — many of them high-tech based — an opportunity to raise funds by trading their shares publicly. Raising funds to commercialize high-tech applications, particularly in biotechnology, has until now been a difficult operation in Europe. European investors have been notoriously conservative when investing in new businesses, considering them to be too risky. Investors tend to like to know when they will see a high return for their initial gamble.

One of the best routes for making profits from an investment is to sell the stake when the company starts trading its shares to the public through one of the stock exchanges. However, the rules to list shares on these exchanges make it difficult for small and new companies to be able to use that route.

It is believed that this lack of funding — a so-called equity gap — has held back the development of entrepreneurial high-tech firms in Europe. By creating a well regulated quality exchange without cowboy companies the EASDAQ founders believe they can tip the high risk/high return balance in favour of the investor. This shift in the balance is then expected to catalyse greater interest in similar companies and ideas.

Mike Ward

... as biotech deal sets record

Oxford. In the largest deal so far involving an entrepreneurial European bioscience company, AgrEvo, a German pesticides producer, has bid US\$550 million to acquire 75 per cent of Plant Genetics Systems International (PGS), the Belgian-Dutch plant biotechnology specialist. The final bill, however, could come to more than \$730 million, as the German multinational plans to acquire the whole company.

PGS has always been at the cutting edge of agricultural biotechnology and possesses a broad portfolio of patents covering important agronomic traits and enabling technologies for genetically modified crops. One of the company's main achievements has been the modification of crops so that they can tolerate the presence of Basta, a broad-spectrum weedkiller developed by AgrEvo that allows farmers to control all weeds in one shot without harming the crop.

It has been estimated that the market for genetically modified crops could reach \$6 billion worldwide by 2005. Although AgrEvo's acquisition of PGS is not that surprising, the price looks very large. Last year, Monsanto acquired a 49.9 per cent

stake in Calgene, the US plant biotechnology flagship company, in a deal that valued Calgene at \$200 million. With PGS having assets of about \$30 million, it can be assumed that AgrEvo estimates the value of PGS technology to be in the region of \$700 million.

Most agrochemicals producers believe that plant biotechnology will underpin future agricultural technology and PGS holds an impressive portfolio of important patents. Although there are clearly synergies between AgrEvo and PGS, other multinational agrochemicals companies are believed to have shown an interest in acquiring PGS and that may have increased the price. This demonstrates, however, how the value of biotechnology companies can be buried in the technology.

Three years ago, PGS attempted to raise funds to move its products into the marketplace by floating itself on the American high-tech stock exchange, NASDAQ, but abandoned the move as it struggled to find buyers. If that flotation had gone ahead, the company would have been valued at about \$200 million.

M. W.

Environment game gives taste of treaties

Boston. Scientists at the Massachusetts Institute of Technology (MIT) have invented a game — the Chlorine Game — that gives students a taste of negotiating an international environment treaty, and is said to point to a better approach to drawing up diplomatic protocols.

The task of the twelve players, representing eight countries and four non-governmental organizations, is to draft a treaty governing the worldwide use of organochlorines. This class of chemicals includes chlorofluorocarbons (CFCs), polychlorinated biphenyls (PCBs) and the oestrogen-mimicking compounds that some blame for declining sperm counts in men.

The game was invented by Lawrence Susskind and his colleagues in MIT's Department of Urban Studies and Planning. Susskind wrote *Environmental Diplomacy: Negotiating More Effective Agreements*, and has advised governments on environmental treaty negotiations.

Students at MIT and Harvard Law School play the game. Several United Nations conferences and centres have also held rounds. The public will get a chance to play later this month when the game is made available through the Program on Negotiation at Harvard Law School.

Susskind says the game shows that treaties could be improved if people discussed the issues informally much earlier on. "Too often people go into these negotiations with specific instructions on what positions to take," says Susskind, who heads the MIT-Harvard Public Disputes Program.

The game also shows the importance of having professional mediators to encourage the dialogue, according to Susskind. The simulation suggests that non-governmental organizations, scientists, and representatives of industry can contribute significantly to negotiations. "But the international community still hasn't figured out how to deal with these people," he claims.

The game is welcomed as a useful training device by Bill Moomaw, a veteran of international treaty deliberations on climate change and ozone depletion who teaches environmental policy at the Fletcher School of Law and Diplomacy at Tufts University, Massachusetts. "If diplomats could play this game before negotiations on organochlorines (or other chemicals) began, they'd have a better idea of how to come up with an effective treaty that doesn't throw out the good, safe chemicals with the bad," he says.

Moomaw also points out that the game has one big advantage over the real world — you can make mistakes without destroying the environment, the economy, or national prestige.

Steve Nadis

Nuclear blast from the past?

London. A recently declassified US military telegram suggests that a fire that occurred in the late 1950s at a nuclear weapons store in the United Kingdom came within a whisker of detonating a nuclear bomb.

The 1956 telegram — from a US military official stationed in the United Kingdom to General Curtis LeMay, commander of US Strategic Air Command — tells how a B47 bomber ploughed into a nuclear weapons store at a Royal Air Force (RAF) base at Lakenheath, Suffolk, setting fire to three 'Mark-six' bombs, similar to that dropped on Nagasaki in 1945. The telegram says that a bomb disposal officer described it as "a miracle" that none of the bombs exploded.

Brief details of the accident were released by the Pentagon in the early 1980s. But this account, and subsequent statements by the UK Ministry of Defence (MoD) have claimed that the bombs did not contain any nuclear capsules. This latest evidence suggests otherwise, says Robert S. Norris, a senior analyst with the Natural Resources Defense Council in Washington. Norris discovered the telegram while browsing through recently declassified material at the US Library of Congress.

Asked to comment on the telegram to LeMay, an MoD spokeswoman says "it is

not up to the MoD to comment on a US document".

Norris believes that officials in Washington would have been similarly notified of two fires — one involving a B47 bomber loaded with a nuclear bomb — that are alleged to have taken place at a weapons facility at Greenham Common in Berkshire in 1957 and 1958. "I don't have that cable, but am certain that one exists."

The latest revelations follow the leaking last week of correspondence from a scientist from the Atomic Weapons Research Establishment at Aldermaston in Berkshire, investigating high levels of enriched uranium beyond the perimeter of Greenham Common in the late 1950s. The scientist suggested that fires could have been the cause. Another incident recorded in a declassified RAF operations record book, reports the accidental jettisoning of a 20–30 kilotonne nuclear weapon from an aircraft onto the hard standing of RAF Wittering in Lincolnshire in May 1959. The accident resulted in "severe damage" to the weapon.

The bomb is understood to have been accidentally dropped between 50 and 100 feet from the ground as the bomber was landing. Informed UK sources say that, while no radioactivity was released, the

bomb's casing was "severely dented". The casing would have been fractured had the bomb been jettisoned from an altitude higher than 1,000 feet.

Last week, the MoD did not rule out accidents involving 'dummy' bombs. But

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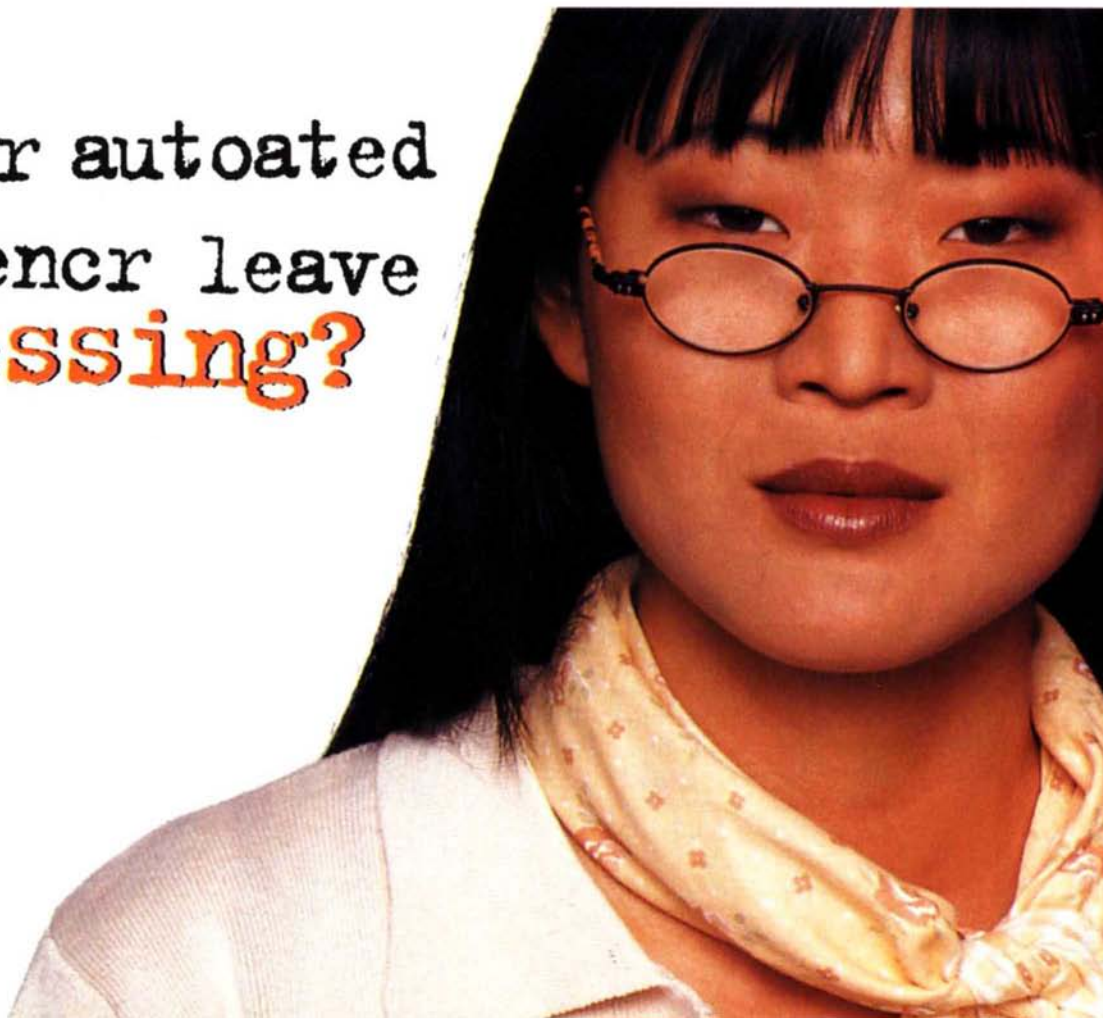
Red alert: a nuclear bomber at Wittering.

sources confirm that the bomb in question was an actual nuclear weapon, a fact borne out by the record book entry, which says the aircraft was returning from 'Exercise Mayflight'. This, says Norris, refers to a drill in which loaded nuclear bombers practised getting off the ground within 15 minutes and heading for a designated airfield in response to a possible Soviet missile attack.

The ministry, while acknowledging that accidents have taken place, continues to insist that none involved the release of radioactivity or posed a danger to the public.

But Rebecca Johnson, of the London-

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based publication *Disarmament Intelligence Review*, believes there has to be some explanation for high levels of enriched uranium around Greenham Common in the 1950s. An accident or fire appears the most likely cause as nuclear weapons were deployed but not manufactured at Greenham Common, she says.

Both Johnson and Norris say the British government should be less secretive about nuclear accidents, in line with the US decision to do so in the early 1980s. "Local residents and the emergency services need to be fully briefed," says Johnson. But such a move is unlikely to be supported within the MoD on national security grounds.

Sir Hermann Bondi, chief scientific adviser to the MoD between 1971 and 1977 suggests that the 'public interest' is not always served best by total transparency. "It is not in the public interest that many people should know the location of a [nuclear weapon] store, or what is in it."

But Johnson says 'politics' rather than national security is the reason why accidents at Britain's nuclear installations remain a secret. The government, she says, was determined to push ahead with deploying nuclear weapons in Britain. But it was under pressure from the newly formed Campaign for Nuclear Disarmament (CND). Any adverse publicity from nuclear accidents would have made the CND's case much stronger, she says.

Ehsan Masood

Panel proposes disarmament guide

Sydney. A report from a high-level international panel of scientists, politicians and diplomats last week not only concluded that the time has come to eliminate nuclear weapons, but proposed a detailed scheme to achieve this.

The 17 members of the Canberra Commission on the Elimination of Nuclear Weapons including Robert McNamara, a former US defence secretary, Michel Rocard, a former French Socialist prime minister, and Joseph Rotblat, president of Pugwash and the 1995 winner of the Nobel peace prize. The leading academic on the panel is Robert O'Neill, an Australian national and now Oxford professor of the history of war. The panel's findings will be presented to the general assembly of the United Nations next month.

The panel calls for "immediate and determined efforts [to] rid the world of nuclear weapons and the threat they pose to it", while warning of "the increasing odds of a calamity" despite the end of the Cold War. It urges the five nuclear weapons states — the United States, Russia, France, China and the United Kingdom — to "commit themselves unequivocally to the elimination of nuclear weapons and to agree to start work immediately on the practical steps and negotiations required for its achievement".

O'Neill describes the panel's recommendations as "the fullest and most radical yet". They include six steps towards disarmament: taking nuclear forces off alert, removing warheads, ending deployment of non-strategic nuclear weapons, banning nuclear tests, further reducing US and Russian nuclear arsenals, and agreement on a "no first use" policy. The group deliberately refrained from setting a timetable for these steps.

The commission says its proposals are realistic, and its report includes 300 pages of technical analysis — of the thorny issue of verification in particular — to support this claim. Whether the report's recommendations will have any practical effect will depend much on mobilising public opinion in the United States after November's presidential election, according to O'Neill.

"The reaction of the US State Department has been good and sympathetic," he says. "They could have chucked it in the bin."

Peter Pockley

Correction

Joseph A. Burns of Cornell University was chairman of the Committee on Planetary and Lunar Exploration (COMPLEX) when its report on Mars missions was being written, and not Ron Greeley, who is the current chairman (see *Nature* **382**, 481; 1996).

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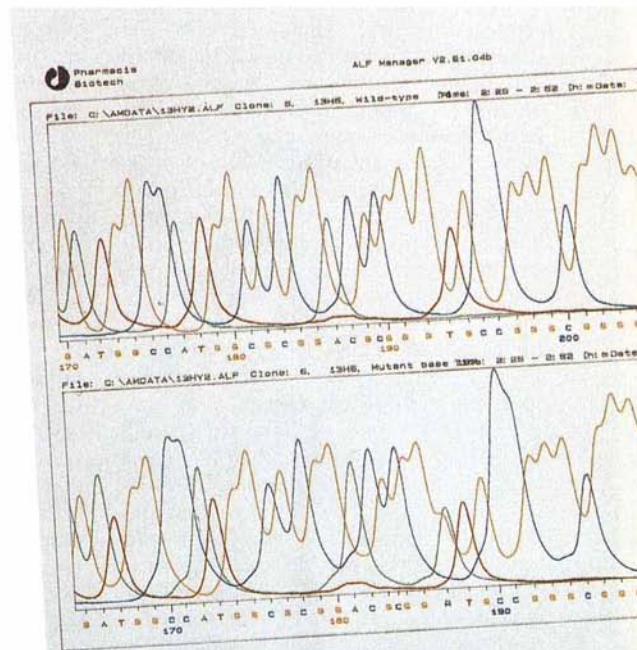
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The p53 gene from 316 breast cancer patients was sequenced using ALF automated sequencing technology. (Bergh J., Norberg, T., Sjögren, S., Lindgren A., Holmberg, L. "Complete Sequencing of the p53 Gene..." *Nature Medicine* 1995; 10:1029-1034.)



Republican platform promises support for biomedical research

Washington. The Republican platform adopted by the party's convention in San Diego last week, called for "private sector funding for applied research," saying that federal science programmes should emphasize basic research. It also promises to "make space travel and space science a national priority".

The document — which Bob Dole, the Republican presidential nominee, says he hasn't read, although it is supposed to guide voters on the priorities of a Dole administration — pledges to abolish the Department of Energy (DoE). It would put the weapons laboratories in "an independent agency under the Department of Defense," and farm out other DoE programmes to other departments and offices.

The platform promises "support for generous funding of medical research", and calls for "fetal protection" in biomedical research. (In 1993, the Democratic Congress passed and President Bill Clinton signed a bill reversing a Reagan-era ban on use of fetal tissue from induced abortions in government-funded research.) □

Japanese climate study satellite

Tokyo. Japan's space agency NASDA last weekend launched its biggest satellite, ADEOS (Advanced Earth Observation Satellite), a platform designed to measure the reduction of the stratospheric ozone layer, and gather information on global warming and climate change. The US\$1.2 billion mission, which was carried out in collaboration with the United States and France, also represents the fourth successful launch of Japan's H-2 rocket. ADEOS is scheduled to operate for two years and to be replaced in 1999 by ADEOS-2. □

German information autobahn

Munich. Germany is to spend DM1.9 billion (US\$1.27 billion) over the next four years upgrading its information and multimedia infrastructure. German participation in electronic publication and global digital libraries are the main goals, according to Jürgen Rüttgers, the research minister. He promises scientists and engineers full desktop access to all available electronic information worldwide by 2000.

Under the programme, a consortium of scientific societies, publishing houses, universities, companies and private users will cooperate with international partners. But the private sector should support information networks and services once the infrastructure is established, says Rüttgers. □

UK pupils flee science

London. The numbers of pupils taking science subjects at Advanced Level continue to decline, according to the results of A-level examinations published last week. The numbers taking humanities subjects remain stable, while those sitting newer and less traditional subjects recorded significant increases.

But the standard of overall performance of the examinations that are taken at the age of 18 registered a sharp rise. This year's pass rate was 85.8 per cent, compared to 84 per cent last year.

Sixteen per cent fewer candidates sat for A-level biology compared to the previous year, 5.65 per cent fewer candidates enrolled for physics and 4.3 per cent fewer for chemistry. Economics also experienced a 7.6 per cent dip in candidates.

Subjects that witnessed an increase include sport/physical education studies (26.6 per cent), media/film/television studies (25 per cent), general studies (10 per cent), business studies (8.4 per cent) and psychology (8 per cent).

The numbers taking mathematics, however, rose by 8.4 per

cent. But this is in part being attributed to the introduction of 'modular' courses, where pupils are examined on specific parts of the syllabus at regular intervals throughout the course, instead of taking one, comprehensive final examination at the end of the two years. □

Pulsar prize

New Delhi. Antony Hewish, a British astronomer, will later this year receive the Indian National Science Academy's Vainu Bappu memorial award, an annual prize named after the late Manali Vainu Bappu, a fellow of the academy and founder of modern astronomy in India. Hewish won the 1974 Nobel prize for physics, along with Jocelyn Bell Bunnell, for his discovery of pulsars. □

US student murders academics

San Diego. A mechanical engineering student at San Diego State University pulled a gun during the defence of his master's thesis last week and shot to death the three professors on his review panel. The three were Chen Liang, Constantinos Lyrantzis and D. Preston Lowry, who was to have become chairman of the Department of Mechanical Engineering next month.

The alleged killer, Frederick Martin Davidson, described as a 36-year-old loner, was arrested shortly after the shooting and charged with three counts of murder. Davidson's thesis had been rejected once before. The killings have left the university community in a state of shock. □

Students 'bought degrees'

Cape Town. Claims that students may have been buying degrees from the University of Zululand, in Kwazulu-Natal, for the past 20 years have led the university council's executive committee to call on education minister Sibusiso Bengu for a government inquiry. An internal inquiry launched by the university following allegations by a student is scheduled to report next month.

A senior administrator at the university, Alson Ngubane, was suspended last week, while 15 students alleged to have obtained degrees fraudulently were barred from graduating. Ngubane claims he is a "sacrificial lamb" and that at least 20 officials and academics were involved. If the extent of the fraud is confirmed, many graduates in senior positions may be found to have bogus qualifications. □

Spanish science head urges caution

Barcelona. César Nombela (pictured), the newly appointed president of CSIC, Spain's central research organization, says he refuses to "dismember" the organization by ceding its research institutes to autonomous regions. Catalonia had demanded such action before the spring general elections.

"We will keep open discussions, but the most we are planning to do is to strengthen our collaboration with local governments, and perhaps open new institutes in the regions where we are not yet present," he says.

Nombela also says he will consider very carefully a proposal by the central government to transfer all government research institutes to CSIC. In May, the government said this should happen within six months, but it backed down following protests from scientists, who argued that research at many of the institutes, including cartography and water management agencies, was not front line and would not fit into CSIC's portfolio. The government has now set up a committee, headed by Nombela, to assess the consequences of merger. □

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Justification of Chapter 8

SIR — The letter from S. Fred Singer¹ continues to spread inaccurate and misleading information about the preparation and approval of the recently published Intergovernmental Panel on Climate Change (IPCC) scientific assessment on climate change². As chairman of the key parts of the Working Group I plenary meeting held at Madrid in November 1995, I should like to explain the IPCC procedures and decisions which led to the acceptance or approval of different parts of the assessment.

At that plenary there were 177 delegates from 96 countries, representatives from 14 nongovernmental organizations and 28 lead authors representing the many hundreds of scientists who had been involved in the preparation and review of the eleven scientific chapters covering 450 pages that form the basic material for the assessment.

The main tasks of the meeting were to approve the detailed wording of the Summary for Policy Makers (SPM) of the report and to "accept" the background scientific chapters for which detailed approval at such a meeting is clearly not possible. According to the rules of procedure, "acceptance" by the plenary means that the meeting is satisfied that they had undergone a thorough process of peer review by experts and by governments and that they present a "comprehensive, objective and balanced view" of the science.

The draft background chapters were sent out to delegates in October in preparation for the November meeting. Subsequently, however, many review comments continued to be received. For instance, the US government, in submitting its points for review, commented on "several inconsistencies" and stated that "it is essential that the chapters not be finalized prior to the completion of the discussions at the IPCC Plenary in Madrid, and that the chapter authors be prevailed upon to modify their text in an appropriate manner following discussion in Madrid".

The Madrid meeting included many hours of discussion and debate about the draft chapter (Chapter 8) on the detection of climate change (which addressed the extent to which anthropogenic climate change has been detected in climate observations), with the object of finding the best and most scientifically accurate wording for the SPM. Unanimous approval both by the scientists and by the government delegates was eventually given to the SPM, which, regarding the detection of climate change, included the following crucial paragraph: "Our ability to quantify the human influence on global climate is currently limited, ... nevertheless, the balance of evidence suggests that there is a discernible human

influence on global climate".

During the plenary sessions, proposals for modification to the draft Chapter 8 were made by both scientists and government delegates. The plenary meeting finally "accepted" the draft chapters (including Chapter 8) subject to their revision by the lead authors to take into account the guidance provided by the meeting and in particular the need for overall consistency. The plenary meeting was, in fact, the final part of the very comprehensive and thorough IPCC process of peer review.

In accordance with IPCC procedures, the subsequent changes to the draft of the chapter were under the full scientific control of its convening lead author, Dr Benjamin Santer. All of us in the IPCC recognize the thorough and careful way in which Santer and his colleagues responded to the proposals made at Madrid. The changes were made with the sole purpose of producing the best possible and most clearly explained assessment of the science and were not in any way motivated by political or other considerations. The IPCC rules of procedure were strictly followed. Not one of the 96 countries represented at Madrid has challenged either the changes in the revised version of the chapter or the procedures.

The IPCC is a scientific body charged with producing scientific assessments. The meeting at Madrid was a scientific meeting whose purpose was to reach agreement on the science of climate change and on the best way of presenting the science to policy-makers with accuracy and clarity. Despite pressure from those with various political agendas, the IPCC has stuck strictly to its brief, refused to compromise its science for any political reason and maintained "complete integrity in the reviewing and approval process", which, as you stated in your leading article³ and was emphasized by Singer, "is an essential element in assuring the credibility of the resulting conclusions".

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only, or e-mailed to corres@nature.com

Hushed tones of the whistleblower

SIR — It is not surprising that "a group of prominent US scientific organizations" opposes the widening of the definition of research misconduct proposed in the report of the Commission on Research Integrity (*Nature* **381**, 263; 1996). The establishment, from which organization chiefs are drawn, is always against punishing the kinds of cheating its members do, typically the theft of credit from underlings and rivals and the misuse of peer review to keep competitors from being funded or published.

Because rank and file organization members are more likely to be victims than cheaters, the organizations' lobbying opposes the interests of their own rank and file. Alas, rank and file members get no chance to vote on the lobbying done in their name.

Ironically, the commission's report, adopted *in toto*, would serve the establishment, because it requires whistleblowers to respect confidentiality. This is not required by current regulation, but whistleblowers are routinely induced to agree to it, and misconduct cases are entirely secret unless the whistleblower wins. The authorities, in thrall to the establishment and unseen by the public, ignore rules and definitions when necessary to do the establishment's bidding. The only weapon whistleblowers have against such misfeasance is to go public. Silencing them by regulation would leave them defenceless.

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The gentle touch

SIR — The protective function of the stapedial muscle has always been puzzling, as it cannot apparently tighten the ossicular chain fast enough for sudden noises, as Daedalus points out (*Nature* **381**, 742; 1996).

In fact, a physiological early-warning system already exists. The stapedius muscle contracts readily to light touch round the meatal entrance, as can be shown with a wisp of cotton wool and a manual acoustic impedance meter, provided middle ears are completely normal. Very occasionally, in some ears, the muscle reflex may be triggered as the wool approaches the ear and before it actually touches the skin. The blast wave from a loud noise or explosion, therefore, may trigger a tactile stapedial reflex in slight advance of the acoustic one, so attenuating the arriving sound.

A. G. Gordon
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Significance of birth-dates

SIR — Various correspondents have commented on the birth-date effect in sport¹, university recruitment^{2,3} and scientific innovation⁴. That particular birth months confer advantage has long been recognized^{5,6}. Recent research has identified their effect as late as the age of 16 (ref. 7) in public examinations in English schools, and it can be seen in the form of selection effects in post-compulsory education at age 18 (data available from the authors) and at degree level⁸. The oldest children in each cohort perform best. Thus Dudink's argument¹ about younger children being disadvantaged is supported by extensive data from large-scale educational assessments.

Maturity apart, various causes of the birth-date effect have been put forward: the effect of climate during pregnancy, the length of schooling effect⁹ arising from varying date of entry into first schools, and each individual's age-position within the year group. The climatic explanation is disproved because international comparisons reveal that birth months conferring advantage vary to follow academic years in countries with differing school/college start dates.

The patterns reported in medical school entry in Italy and Portugal buck the general trend but have to be compared with contrary evidence from large surveys involving thousands of individuals (in some cases entire cohorts). How much weight should be attributed to the evidence of two small sets of data based on nonrandom samples and subject to uncontrolled selection biases?

Age-position effects could explain the pattern in the birthdays of scientific revolutionaries. Could being the eldest and most dominant in their cohort have given some scientists the confidence to challenge established paradigms?

John F. Bell

Alf Massey

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Universal spelling

SIR — Searching the bibliographic database, rather than manually inspecting tables of contents, is becoming common, and the use of both US and British spelling for the same technical term can cause confusion.

I recently submitted a paper about disulfide bonds to a journal published by a British publisher. The technical editor corrected 'disulfide' to 'disulphide' throughout my manuscript. This did not bother me, as scientists recognize the equivalence of these terms. But I soon realized that this causes a problem in database searches.

My searches (24 July 1996) into the Medline Subset in Molecular Genetics via the Internet yielded the following results. Searching for 'disulfide' against the title field and '1996' against the publication year yielded 19 articles, whereas 'disulphide' yielded 4 articles. When the abstract field was selected, these numbers were 117 for disulfide and 18 for disulphide. Most importantly, the papers retrieved by 'disulfide' and 'disulphide' were completely non-overlapping. Unless one repeats searches using both US and British spelling, it is easy to overlook important references. In a field such as biology, insisting on a spelling tradition does not make sense.

My simple-minded proposal for a solution would be to stop using the British spellings (or the other way around, but the figures suggest that this is less feasible) in scientific publications. This may be difficult for British publishers to accept, but they should remember that the rest of the world is forced to use English regardless of our mother tongues.

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Optical patterning

SIR — The technique of optical patterning will be an important step forward in the manufacture of liquid crystal displays (LCDs); but I believe that Schadt, Seiberle and Schuster (*Nature* **381**, 212–215; 1996) overemphasize its importance in improving the viewing characteristics of LCDs.

There are at least three factors that together make a displayed image viewable: brightness, image contrast and, in some cases, colour fidelity. Current LCD technology has problems with all three of these factors at wide viewing-angles. Although the multidomain technique discussed by Schadt *et al.* does improve the brightness distribution by averaging out the light output, it also reduces the head-on contrast. It averages out the relatively high head-on

contrast of LCDs with the poorer contrast at wide viewing-angles.

A comprehensive paper by H. Hatoh (*Proceedings of Semicon/Korea 96*, Flat Panel Display Seminar, pp 35–46, 24 January 1996) evaluated a dozen distinct techniques and their variants for improving the wide viewing-angle performance of twisted nematic LCDs. This evaluation included the multidomain technique discussed by Schadt *et al.* The discussion in Hatoh's paper cites the drawbacks to the multidomain technique, including optical artefacts (disinclination lines) and degrading of contrast ratio. Other techniques discussed by Hatoh gave much better optical performance, including 'in plane switching' and 'diffusing screen system' using a collimated light source. The latter he described as having a "perfectly [*sic*] wide viewing-angle".

Although all techniques have some drawback in practical application, the validity of a technology cannot be measured by one narrow aspect. Brightness distribution is important for displays; but so is contrast and colour fidelity.

Norman Hairston

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Faith, hope and taxation

SIR — George Marsden's review of *The University in Ruins* by Bill Readings (*Nature* **382**, 219; 1996) draws attention to an Oxford college that includes in all its job advertisements a statement of its dedication to excellence. He goes on to point out the emptiness of such statements.

Readers of the Classified pages will have noticed over recent years the growth in such statements in advertisements for British academic posts. In the same issue, one could read of universities "promoting excellence in teaching, learning and research" (three different universities), "at the leading edge of research, innovation and learning", "engaged in teaching and research of international distinction", "dedicated to excellence in teaching and research" and "promoting educational opportunity and the application of knowledge".

The mundane explanation for these ringing affirmations is that universities have discovered that, by some quirk of VAT (value-added tax) legislation, if a charitable (not-for-profit) body includes a brief statement of its aims in advertisements, it need not pay VAT on the advertising bill. Maybe the university's aims should include "excellence at avoiding VAT".

David Davies

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Unpalatable evolutionary principles

Tim Guilford and Candy Rowe

Experiments involving rye straws and almonds as prey, and great tits as predators, unveil forces operating on the origin of warning signals in nature.

EVOLUTION covers its tracks nowhere more thoroughly than in the field of animal signalling, where layers of mutual co-evolution between signallers and receivers obscure the biologist's view of evolutionary origins. On page 708 of this issue, however, Alatalo and Mappes¹ describe experiments that succeed in uncovering selective forces involved in the initial evolution of anti-predator warning signals, by re-creating the starting conditions in a world of novel artificial prey.

Alatalo and Mappes attack a long-standing controversy: many species of toxic insects signal their toxicity to predators with conspicuous colour patterns (they are said to be 'aposematic'), and also live gregariously in family groups — the distinctively yellow-and-black cinnabar moth caterpillar is a classic example (see photograph). Fisher² noticed this association and first suggested that kinship groups might favour the evolution of such costly defences because an attack on one individual by a naive predator could lead to subsequent avoidance of nearby relatives, likely also to carry genes for the defence (an idea that prefaced Hamilton's theory of inclusive fitness³).

But the association itself is not proof, because aposematic prey could have evolved gregariousness secondarily because they have less need to be solitary than did their palatable, concealed (cryptic) ancestors. Furthermore, phylogenetic analysis does not make it clear that gregariousness always precedes aposematism⁴; individual aposematic prey can survive encounters with naive predators (reviewed in ref. 5), and experiments on the role of prey aggregation on predator-avoidance learning have produced contradictory results^{6,7}. The problem of whether gregariousness favoured the initial evolution of aposematism remains because, as Alatalo and Mappes point out, even naive predators are not naive in the evolutionary sense. They may have co-evolved instinctive responses for dealing sensibly with aposematic prey on first encounter. Evolution covers its tracks.

Alatalo and Mappes have got round the co-evolutionary problem by presenting

birds with an entirely different world of prey that do not use colour signals at all, but black-and-white printed symbols glued like little flags to the ends of a hollow straw filled with edible fat. Prey were made cryptic or conspicuous by a background of like or unlike symbols. As the prey signals and their backgrounds were entirely novel, the authors were free to use real insectivorous predators without worrying about the predators' previous ex-

new unpalatable species, gregariousness enhanced the effectiveness of the toxic defence. Third, and perhaps most important, the conspicuous unpalatable morphs survived far better in the aggregated treatment than in the solitary treatment, where they suffered an initial increase in predation.

Initially, then, gregariousness enhances the effectiveness of unpalatability, and provides the conditions under which a conspicuous warning signal is evolutionarily advantageous right from the start. What happens once warning signals become established? Now the selective conditions appear to change. In a second experiment with the same birds, Alatalo and Mappes introduced a new prey type, slices of almond (some naturally palatable, some made unpalatable with chloroquine) with the little flag symbols, which were distinctive in shape but carried the same warning signals with which the birds had become familiar. They were imperfect mimics. Now gregariousness seemed to have little effect on the survivorship of unpalatable prey, suggesting that once aposematism is established, other toxic prey species may readily evolve similar warning signals (Müllerian mimicry) without the help of a gregarious lifestyle.

Alatalo and Mappes have succeeded in showing us that the effect of aggregation on predator avoidance of warning signals is not merely a secondarily coevolved response to the fact that nature is already full of nasty, brightly coloured, aggregated prey. It is more deeply rooted than this, operating in a novel world now, and presumably then, when insect defences were first evolving. By stripping back the co-evolutionary veil, they have exposed evolutionary forces operating on

IMAGE
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FOR REASONS
REASONS

United in bad taste — a group of caterpillars of the cinnabar moth (*Tyria jacobaeae*) send vivid signals of their toxicity to potential predators.

perience or evolutionary involvement with aposematic prey. Great tits (*Parus major*) were caught in the wild and released into a laboratory containing cryptic, palatable prey (on which the birds had already been trained) and a new 'species' of unpalatable prey with half the morphs cryptic and the other half conspicuous. The important manipulation, though, was to present half the birds with solitarily arranged prey and the other half with gregariously arranged prey.

There followed three important discoveries. First, birds showed no innate aversion to aggregated prey, because palatable prey in the aggregated treatment were even more vulnerable than in the solitary treatment. Second, unpalatable prey were initially less vulnerable to predation in the aggregated than in the solitary treatment. Even for the cryptic morphs of the

Heather Angel

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the origin of defensive strategies. This part of the controversy seems solved: aggregation enhances initial avoidance of unpalatable prey, so aposematism is more likely to have evolved in aggregated species initially. The subsequent evolution of mimicry is not so constrained.

Yet we suspect that the controversy is not altogether over. Because the enhanced protection of aposematic prey in

aggregations is only an initial effect, it is possible to imagine conditions under which a new toxic or warning-coloured morph might reach sufficient abundance to bypass the need for this initial boost to its fitness (perhaps predators are rare one year). Similarly, even once aposematism is established, conditions may sometimes appear in which there are so many naive predators that gregariousness may

provide significant benefits to fitness. A little stochasticity in nature may have allowed evolution partially to cover its tracks again, and the controversy to rumble on. □

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AIDS

Natural resistance to HIV?

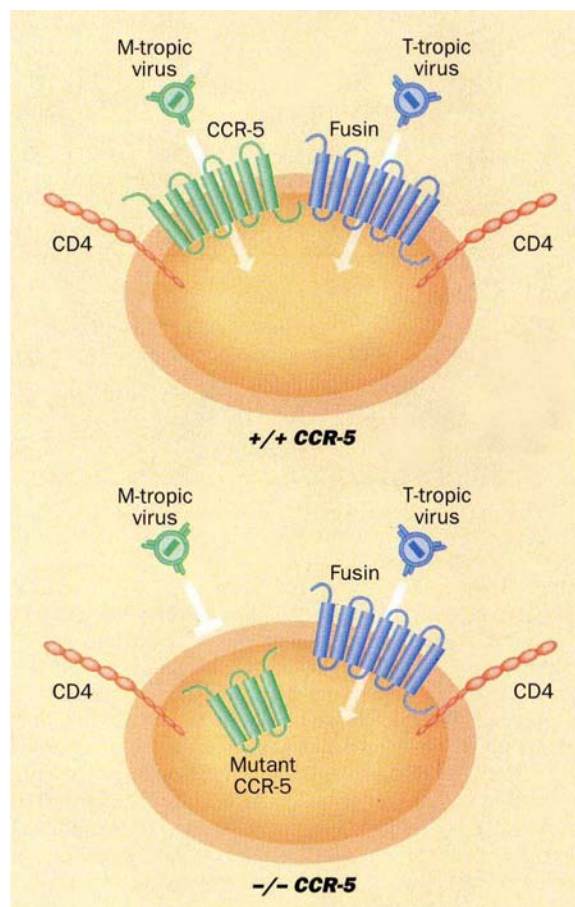
C. Mark Hill and Dan R. Littman

EARLIER this year, Paxton *et al.*¹ described several individuals who had remained free from infection with the human immunodeficiency virus (HIV) despite repeated exposure through sexual intercourse with HIV-positive partners. When HIV was applied to white blood cells (leukocytes) from these exposed-uninfected subjects, it was found that cells from two of them were highly resistant to infection with strains of virus that are believed to be important in person-to-person transmission. This remarkable observation dovetailed nicely with the subsequent discovery of several cell-surface glycoprotein cofactors that cooperate with the CD4 molecule to permit entry of HIV into cells²⁻⁷.

These findings have been brought together by Liu *et al.*⁸, who in a paper published earlier this month in *Cell* show that the two exposed-uninfected subjects harbour identical mutations, on both chromosomal copies, of one of these cofactor genes. On page 722 of this issue, Samson *et al.*⁹ identify the same mutation and describe its prevalence in HIV-infected and uninfected populations in Northern Europe. The mutation is the most likely explanation for how the exposed-uninfected individuals have resisted the virus, and also provides important clues as to the mechanism by which HIV gains a foothold in those who are exposed to it.

The key cofactor is CC-chemokine receptor 5 (CCR-5), a member of the seven-transmembrane, G-protein-coupled receptor family¹⁰. In response to the CC chemokines called RANTES, MIP-1 α and MIP-1 β , this receptor transduces signals in leukocytes that result in their chemotaxis towards areas of inflammation. Late last year, Cocchi *et al.*¹¹ showed that these chemokines could also inhibit infection by several primary strains of HIV-1 that target macrophages and CD4⁺ T-helper cells (M-tropic strains); this effect was explained by the later demonstration that CCR-5 mediates entry of these strains of HIV-1 into cells following binding of their envelope glycoproteins to CD4 on the cell surface. HIV-1 strains that

infect CD4⁺ T-helper cells, but not macrophages (T-tropic strains), utilize a related putative chemokine receptor, fusin or LESTR, and are not inhibited by the CC chemokines. Although most HIV-1 isolates studied to date utilize CCR-5 or fusin, some can use both as well as the related CCR-2B and CCR-3.



HIV infection of target cells is dependent on CD4 and chemokine receptors. Macrophage-tropic (M-tropic) viruses bind to CD4 on the surface of target cells via their envelope glycoproteins. Entry of the virus into the cell is then dependent on the CC-chemokine receptor CCR-5. Similarly, T-cell-line tropic (T-tropic) viruses bind to CD4 and are then dependent on the CCR-like molecule fusin for entry into cells. In people who are homozygous for a mutation in CCR-5, T-tropic viruses can still enter target cells via fusin, but entry of M-tropic viruses is impaired.

Liu *et al.*⁸ and Samson *et al.*⁹ identify a mutant allele of CCR-5 that is homozygous in the two exposed-uninfected individuals described above. This allele contains a 32-base-pair deletion that results in a truncated protein which fails to mediate chemokine signalling and HIV infection *in vitro* (see figure). The implication of these data is that CCR-5 is a major determinant for HIV infection *in vivo*. Surprisingly, both groups found the polymorphism to be quite common in caucasians of European descent, with an allele frequency of 0.092–0.098.

About 15–20% of the caucasian population are heterozygous for the mutation, while about 1% are homozygous.

Consistent with the prediction that absence of CCR-5 confers protection from HIV-1 infection, Samson and colleagues⁹ found no homozygous mutants among 723 HIV-1 infected subjects, but detected eight among 704 uninfected individuals. Intriguingly, they also found a reduced frequency of heterozygotes with a mutation in CCR-5 in the HIV-infected compared to the uninfected population. This suggests that there may be partial protection from infection in people with a single copy of the mutant gene. Both groups, however, clearly demonstrate that cells heterozygous for the CCR-5 mutation are susceptible to viral infection, albeit at reduced levels. Moreover, the populations compared were not ideally matched, and there may be significant variations in allele frequencies even between different regions in Western Europe. It therefore remains unclear if either transmission of HIV or progression to AIDS are affected in individuals heterozygous for the CCR-5 mutation.

Given the limited number of subjects examined in these studies, it is also too early to conclude that a lack of functional CCR-5 predicts protection from HIV infection. For example, other chemokine receptors may be used by some strains of HIV-1 or may be favoured by non-sexual routes of transmission. It is also possible that exposed-uninfected individuals have other genetic factors that, along with the CCR-5 polymorphism, have contributed to their protection from infection. Large-scale studies with carefully matched cohorts will be required to clarify these issues.

The potential importance of CCR-5 in viral transmission is consistent with previous findings that M-tropic viral strains predominate shortly after infection¹². Because individuals are probably exposed to both T-tropic and M-tropic strains of HIV-1, it remains puzzling as to why viruses that use other cofactors (fusin, for example) are not transmitted. The mechanism of transmission apparently cannot involve only CD4⁺ T-helper cells, which express both fusin and CCR-5. Primary infection may depend upon cell types that only express CCR-5, most likely cells of the macrophage lineage. In addition, in particular cell types CCR-5 may be endowed with unique intracellular signalling properties that are required for establishment of infection.

These two new papers^{8,9} describe the first human genetic polymorphism that may affect the spread of HIV-1. However, only two of the 25 exposed-uninfected subjects studied by Paxton *et al.*¹ were found to be resistant to infection *in vitro* and to lack functional CCR-5. It will be interesting to learn whether additional genetic factors can provide resistance to infection by different mechanisms, such as regulation of chemokine production or of interactions between cells in the immune system. There are also numerous individuals who do not develop symptoms of disease even after being infected for many years. Do these long-term 'nonprogressors' have a genetic predisposition that protects them from the pathogenic effect of the virus? Studies of other chemokine receptors, such as fusin, which is utilized by viral strains that accumulate late in the disease process¹³, may provide

further insight into disease progression.

People lacking functional CCR-5 do not appear to be immunologically compromised, although this has yet to be studied in detail. The frequency of homozygotes versus heterozygotes with the mutation fits what would be predicted if the mutation is not significantly deleterious in the present-day caucasian population. The mutant allele has not been found in the limited number of African, Venezuelan or Japanese subjects examined to date, suggesting that this specific polymorphism arose recently in caucasians. One can speculate that the mutation may have conferred a protective advantage on part of the caucasian population, possibly in response to an HIV-related virus or other pathogens.

The absence of any obvious physiological defects caused by the CCR-5 homo-

zygous mutation also points to potential strategies for treating HIV infection without compromising the immune system. Specific antagonists of CCR-5-mediated infection could mimic the effect of the CCR-5 null mutation and hence would not be deleterious. Discovery of a human genetic polymorphism in CCR-5 is a big step in our progress in understanding the critical relationship between chemokine receptors and HIV infection. But it is important to emphasize that it is still too early to conclude that this mutation alone confers any natural resistance to infection by HIV. □

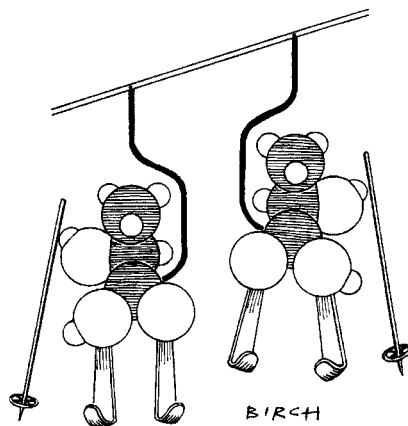
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INFORMATION THEORY

Freely communicating

Charles Bennett

ROLF Landauer, responsible more than anyone else for establishing the physics of information processing as a serious scientific discipline, often feels called upon to clear up misconceptions in the field. In a paper in *Science* on 28 June¹, he seeks to refute the notion that information transmission has an irreducible thermodynamic energy cost per bit transmitted.



An energetically efficient communications channel based on the transport of left- and right-handed lactic-acid molecules.

This belief arose from Shannon's proof² that $kT \ln 2$ of energy (Boltzmann's constant $k \approx 1.38 \times 10^{-23} \text{ J K}^{-1}$, and T is absolute temperature) is needed to make signals in an electromagnetic communication channel distinguishable against the unavoidable background of thermal noise. Despite its practical importance, Shannon's bound does not apply to all types of channel. In particular, Landauer points out, it does not apply when the internal

state of a material body is used to carry information from one place to another. Although such channels—the postal service, for example—are often slow and energetically inefficient in practice, in principle they can be made highly efficient.

In assessing the energy cost of communication, one must distinguish permanent losses (energy turned irreversibly into heat each time the channel is used) from temporary energy investments, such as the kinetic energy of an information carrier while it is in motion from sender to receiver, which in principle can be recovered and re-used. It is not fully understood under what circumstances the signal energy in an electromagnetic channel can be re-used, but when a material body is used as the information carrier, both the energy investment and the energy loss can be made arbitrarily small.

The heart of Landauer's energetically efficient communications channel is an intrinsically bistable material body with two low-energy states separated by a potential-energy barrier high and broad enough to effectively prevent both thermal hopping and quantum tunnelling between the states. He uses the abstract model of a bistable potential well, but a more concrete example would be a simple chiral molecule such as lactic acid ($\text{CH}_3\text{-CH(OH)-COOH}$), whose right- and left-handed forms can be used to encode the binary digits 0 and 1. Information can be loaded and unloaded from such a carrier at the sending and receiving ends by reversible stereospecific chemical reactions such as those of natural lactic-acid metabolism. These reactions are reversible in

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the thermodynamic sense that the energy dissipated per bit loaded or unloaded tends to zero in the limit of zero speed.

To transport his bistable systems, Landauer invokes the technology of a modern ski-lift, which accelerates its passengers smoothly from a standing start at the boarding point, then drops them off equally smoothly at their destination. Such a system, also reversible in principle, could transport molecules or other bistable systems without risking inelastic effects that might be caused by a sudden change of velocity.

Once it has been brought up to speed, the molecule has kinetic energy and internal excitation energy above the ground state (which is a symmetric superposition of the two chiral states). Both are re-usable, but in any case they can be made negligible compared with kT by moving the molecule slowly and by choosing one with a sufficiently large potential-energy barrier.

Ordinarily, one thinks of matter as an available building material, but if one had to manufacture a material information carrier out of nothing, the energy investment needed would be the carrier's rest energy, $E=mc^2$. Although far larger than

kT for any molecule stable at temperature T , rest energy is almost perfectly re-usable, because spontaneous processes that would dissipate it (proton decay or cold fusion, for example) are rare even on cosmological timescales. By re-using the carrier more than mc^2/kT times, the rest energy per bit transmitted can be made less than kT .

Although molecules are quite stable against proton decay and cold fusion, they are less stable against loss of their stored data via tunnelling through or hopping over the energy barrier. That sets a limit on how slowly the molecular channel can be operated, which in turn sets a minimum (typically much less than kT , but still greater than zero) on the dissipative losses from chemical reactions, which approach zero only in the limit of zero speed. Fortunately, one can make the energy barrier higher and broader by using larger molecules, increasing the half-life of stored information roughly exponentially with molecule size. Meanwhile, the chemical reaction times also increase, but much more slowly. That suggests that there is no limit to the energy efficiency of matter-based communications channels other than those set by the stability of matter itself.

In the 35 years since Landauer's early paper³ on irreversibility and heat generation in the computing process, the thermodynamics of information processing has become well understood⁴. General-purpose computation, communication, and even measurement can be performed in a thermodynamically reversible fashion, so long as information is not thrown away; in other words, so long as there is a 1:1 mapping between the initial and final states of the information-processing apparatus. If information is thrown away, the energy cost is $kT\ln 2$ per bit discarded.

In the past few years there has been a belated realization that the laws of quantum mechanics give rise to a larger and more complete theory of communication and computation, encompassing the transmission and manipulation of intact quantum states as well as classical bits (see refs 5 and 6, and reprints in the Los Alamos archive⁷). This larger theory explains and predicts such novelties as quantum cryptography, quantum data compression, the massive speed-up of certain computations by quantum parallelism, and, most recently, quantum error-correcting codes and fault-tolerant quantum gate arrays, which raise the hope of achieving quantum computation in practice.

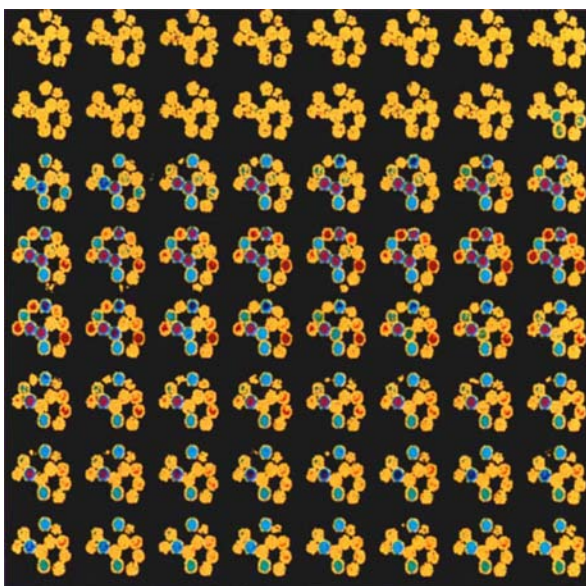
Landauer's main contribution to this explosion of interest in quantum computation has been to offer constructive criticisms and warn against excessive optimism, but his molecular ski-lift, perhaps inadvertently, makes use of some of the new theory's most fashionable features. Via the so-called quantum watchdog effect, even extremely weak interactions between the lactic-acid molecule and its environment serve to stabilize the molecule's left- and right-handed states, while greatly accelerating the decay of any coherent superposition, such as the isolated molecule's true ground state. These interactions, in other words, make the lactic-acid molecule a better carrier of classical information, but useless as a carrier of quantum information. Isolating the molecule from the interactions (very difficult in practice), or using a quantum error-correcting code to hide a one-bit quantum message in a redundantly entangled state of five molecules, would allow the channel to be used for both kinds of information. □

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SENSORS

A triumph for common scents

THE smell of benzene is a familiar one to organic chemists, yet it has never appeared like this before. This time-series of images of fluorescent intensity from the tips of a bundle of optical fibres encodes a spatiotemporal 'fingerprint' of the organic compound. In the artificial nose described by Walt and co-workers on page 697, a neural network has been trained to recognize this fingerprint. Each fibre tip is coated with a dye and one of several different polymers; the colours represent the fluorescent response of each dye/polymer combination to a pulse of benzene gas (yellow is the resting state, and green, purple and red indicate increasing intensity; blue indicates decreased fluorescence). Images of the response (here taken at intervals of 133 milliseconds) together provide a unique time-varying signal for many different odorant molecules. An artificial nose of this sort mimics the operation of



the olfactory system, in which an array of cross-reactive odour receptors or sensors provides a complex signature of each odorant, which is then decoded in a higher-level processing operation than the detection events themselves. A system of this sort, which sniffs out low concentrations of chemical species with high selectivity, might find valuable uses in environmental and medical monitoring.

Philip Ball

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Plastic lasers shine brightly

Donal Bradley

LASERS have progressed rapidly from being a solution in search of a problem to being a widespread part of modern technology, used in compact disc players, optical-fibre-based telecommunications and laser surgery. Lasers also offer a fascinating view of the quantum world, in which photons act as particles and interact in a sheep-like manner to produce concerted emission, with the special properties of coherence, directionality and high intensity. The search for new lasing media to extend the wavelength range and to reach new temporal regimes (in pulse profile) has been a consuming passion for several decades. Now Nir Tessler, Graham Denton and Richard Friend (on page 695 of this issue¹) describe experiments that indicate lasing in a conjugated-polymer microcavity. This is a new class of medium for compact laser sources (in their miniature device the active volume of polymer is only $5 \times 10^{-9} \text{ cm}^3$).

Polymers are chain-like organic molecules that consist of many individual segments, or repeat units, linked in a well-defined sequence. The conjugated polymers are a subset of these materials in which electronic states can delocalize, allowing stable optical excitations (without bonds being broken) and mobile charge carriers. They have attracted widespread attention over the past twenty years, first as 'synthetic metals' (man-made materials of high conductivity) and increasingly as semiconductors for electronic, optoelectronic and nonlinear optical applications.

Their emissive properties are particularly attractive, especially when combined with low materials costs and the possibility of large-area device fabrication. The property of electroluminescence, in which light emission follows the combination of electrons and holes injected from electrodes in a diode structure, is the subject of intense research and development worldwide. Emission across the whole of the visible spectrum has been demonstrated, and efficiencies, brightnesses and device lifetimes are rapidly approaching commercially useful target figures for lighting and displays².

One way to optimize the performance of electroluminescent diodes is to sandwich a polymer film between two mirrors. When the film thickness is of the order of the wavelength of the emitted light, the structure acts as a planar microcavity resonator. This structure couples the emitting dipoles in the polymer film to an electromagnetic standing wave, and allows control of the emission line width, directionality and decay rate.

The microcavity resonator is also a structure that lends itself to the attainment

of stimulated emission and lasing. Certain types of inorganic semiconductor laser already make use of just this device configuration. But can it be done with the more versatile conjugated polymers?

In their article¹, Tessler *et al.* study the properties of a polymer-based microcavity (see Fig. 1 on page 695) under intense optical excitation, or 'pumping'. They observe three intensity-dependent phenomena with a clear threshold behaviour. First, at high pump intensity the device

A plastic laser. This 1-cm-thick cuvette contains a conjugated polymer solution which lases at 446 nm. Amplification occurs even without an external cavity. Widely tunable lasers may be possible in the future, using solid films of this and other polymers.

selects a single specific mode to which the emission strongly couples — this occurs at the expense of two other low-intensity cavity modes. Second, the emission lines narrow and third, the directionality increases, so more light comes out normal to the mirror surfaces. Taken together, these characteristics are strong evidence for lasing.

Organic dye solutions are used widely as tunable lasers, and lasing with conjugated polymers has been seen before in dilute solutions where the polymers mimic conventional laser dyes³⁻⁵, but solid films were thought to be unusable because of strongly competing induced absorptions that kill net gain⁶. The article of Tessler *et al.* argues against this perceived wisdom, and is supported by recent experiments on thin polymer films, both undiluted⁷⁻⁹ and containing TiO_2 nanocrystals¹⁰. These films also show strongly narrowed emission spectra and increased intensity above a well defined pumping threshold. But in all these cases some caution is necessary in concluding that lasing occurs. Super-radiance and superfluorescence are phenomena that involve collective excitations of an ensemble of dipole-coupled molecules. Both of these increase intensity and produce emission-line narrowing above a specific threshold of excitation density, mimicking laser action.

Whether such phenomena play a role here remains to be seen. But note that the solid-state photoluminescence quantum yields of 70 per cent and higher indicate that lasing should be possible. Whatever the conclusion, it is clear that conjugated polymers are now entering a new phase of study as highly emissive solid state materials. That is important for understanding their fundamental photophysical processes, and it may also open new ways of studying collective phenomena shown by excitons (bound electron-hole pairs) at high excitation densities.

For most purposes, electrical pumping would be much more convenient than optical. Low-cost electrically pumped lasers operating at wavelengths across the visible and near-ultraviolet spectral ranges would be very attractive if good power conversion efficiencies could be achieved. Tessler and colleagues' microcavity can be modified for electrical excitation by inserting a transparent anode (indium tin oxide, for example) on top of the Bragg reflector. Practical devices look a long way off at the moment because simple estimates¹⁰ suggest that current densities of 10^5 to 10^6 A cm^{-2} may be needed at present to reach the threshold excitation density achieved

by optical pumping, but with foreseeable improvements in performance that figure might well be drastically reduced. It is also possible that some of the features of optically pumped conjugated-polymer lasers may prove attractive in their own right. For example, wide tuning ranges are anticipated⁵, which would mean that a single polymer could replace several more conventional dyes.

Whatever the outcome of this new chapter in conjugated polymer research, the chase will certainly be fun. Many surprises undoubtedly lie in wait on the way. □

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X. Long & D. Lidzey

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REASONS

What's mice got to do with it?

Kenneth W. Kinzler and Bert Vogelstein

LESS than two years ago, a fiercely competitive race to find one of the genes responsible for hereditary breast cancer ended with the discovery of *BRCA1* (ref. 1). As is not uncommon, the sequence of the new gene provided few insights into its function. Thus began a new race to define the function of the gene product, a race that has turned out to be even more competitive and controversial than the last.

Among the newest entries in this contest are mouse 'knockouts' devoid of the *BRCA1* gene, which are described in *Nature Genetics* by Gowen *et al.*² and in *Cell* by Hakem *et al.*³. Somewhat surprisingly, *BRCA1*-null mice die during early embryogenesis, but they have already provided some new clues consistent with the idea that the *BRCA1* protein is a transcription factor that puts the brakes on cell growth. This proposal is neatly supported by results from Chapman and Verma, reported on page 678 of this issue⁴, which reveal that the carboxy terminus of *BRCA1* contains a domain that can activate transcription.

The embryonic death of the homozygous knockout mice was unexpected because humans with homozygous mutations of *BRCA1* are capable of normal growth and development, with their only defect being a predisposition to breast and ovarian cancer similar to that found in heterozygotes⁵. In contrast, the heterozygous mice have no apparent predisposition to breast cancer (at least by one year of age; refs 2 and 3, and T. Mak, personal communication). Although residual activity of alternatively spliced forms of *BRCA1* might account for the viability of human homozygous mutants, the most obvious implication of these results is that *Mus musculus* and *Homo sapiens* are not the same beasts, a detail that often seems more obvious to our children than to us 'professionals'. Previous knockouts of cancer-related genes have, in fact, usually revealed phenotypes in mice different from those expected from the analogous humans. Some tumour-suppressor-gene knockouts in mice result in no tumour predisposition (for example, *WT1*), whereas many others result in tumours of cell types that are significantly different from those observed in humans with inherited mutations of the same genes.

In the light of these substantial differences, are studies of mouse knockouts useless, or worse, misleading, for our understanding of human cancer? Certainly not. Although the series of events resulting in phenotypes as complex as neoplasia cannot be expected to be equivalent in mice and humans, the interactions between individual gene products and the biochemical pathways connecting them

are likely to be well conserved. Murine cells with disrupted genes thus offer unique opportunities for studying such pathways in a highly controlled fashion.

Mice with disrupted *p53* genes, for example, have provided unequivocal evidence that *p53* protein is required for the normal response to agents that induce cell-cycle perturbations, a hypothesis that could only be suboptimally tested before the creation of these mice. In mouse cells without *BRCA1*, there are substantial changes in the expression of the *p53*-regulated genes *p21* and *mdm2* (ref. 3). This connection between the pathways controlled by *BRCA1* and *p53* is supported by independent studies demonstrating a striking coincidence between the cell-cycle distributions of *BRCA1* and *p53* proteins^{6,7}. Interestingly, inherited mutations of *p53* result in a predisposition to breast cancer in humans (but not in mice!).

How do the observations in *BRCA1*-null mice jive with previous studies of *BRCA1* function in human cells? Some recent observations support the idea that *BRCA1*, like *p53*, is a transcription factor, but the data are somewhat contradictory. Most have focused on the intracellular localization of the gene product. The first clue was provided by an immunohistochemical study carried out by Chen *et al.*⁸, which showed that the *BRCA1* protein was normally found in the nucleus. This observation is now significantly extended by Chapman and Verma⁴, who demonstrate that the carboxy terminus of *BRCA1* contains a transcriptional activation domain; this transcriptional activation capacity was markedly diminished in each of four mutant *BRCA1* proteins derived from patients with hereditary breast cancer.

Chen *et al.*⁸ reported that the localization of *BRCA1* was often aberrant (in the cell cytoplasm instead of the nucleus) in non-hereditary breast cancers. This observation was exciting, because it suggested that *BRCA1* might play a role in non-hereditary breast cancers (which account for more than 90 per cent of the total breast cancers in the Western world) as well as in the hereditary types. But, alas, the excitement was short-lived, as other investigators, using similar immunohistochemical techniques, concluded that *BRCA1* was sited in the nucleus in both normal tissues and non-hereditary breast cancers⁹.

Further complicating the idea that *BRCA1* encodes a transcription factor is a report suggesting that *BRCA1* is not nuclear at all, but rather is a secreted protein localized in secretory vesicles¹⁰. This was provocative, in part because it might explain one of the most puzzling aspects of

BRCA1 epidemiology. Whereas inherited mutations of *BRCA1* lead to predisposition to breast and ovarian cancer, acquired *BRCA1* mutations are extraordinarily rare in sporadic (non-hereditary) breast or ovarian cancers. This is in stark contrast to most previously described tumour-suppressor genes, in which somatic mutations result in sporadic cancers of the same type.

This conundrum might be explicable if *BRCA1* were secreted, thereby precluding a cell-autonomous effect on neoplasia. However, because breast lobules normally develop in a monoclonal fashion¹¹, one can imagine that a somatic mutation of *BRCA1* could initiate neoplasia if it occurred in a lobule with a pre-existing hereditary mutation of the other allele.

These conflicting results illustrate the power of knockout mice to resolve fundamental questions about cancer genes. For example, nuclear (cell-autonomous) and secreted (non-autonomous) models of *BRCA1* function predict dramatically different phenotypes in experimental situations, particularly with chimaeras between wild-type and knockout mouse embryonic stem cells. Additionally, a major problem for cell localization studies is that an antibody's specificity as indicated by immunohistochemistry cannot necessarily be inferred from its reactivity in western blots or immunoprecipitation¹².

What is needed to guarantee specificity are perfectly matched controls, in which endogenous levels of the relevant protein can be assessed immunohistochemically. Mouse cells with disrupted *BRCA1* genes can provide such controls: absolutely no reactivity with the antibody should be detected in such cells, in contrast to otherwise isogenic cells of the same subtype. Although analogous controls may be furnished by breast tumour cells from humans with hereditary *BRCA1* mutations¹³, the mice provide a more reproducible and manageable source of such reagents. With the availability of mouse knockouts, it therefore should not be long until we know where the *BRCA1* gene product really lives, at least in mice. □

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Beauty stays as charm wilts

David J. Miller

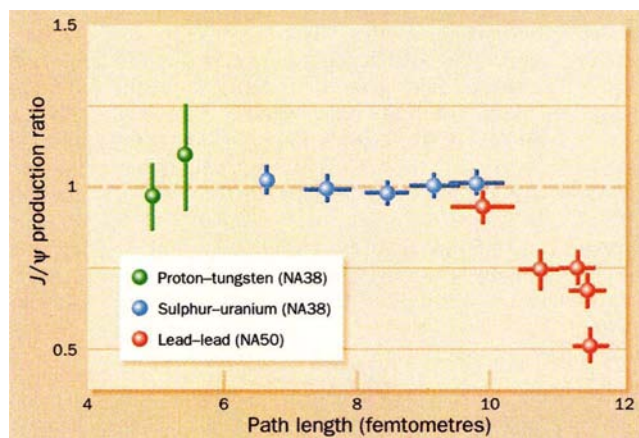
SOME said, before last month's Warsaw meeting*, that 1996 would be the year we began to believe in the theory of supersymmetry. Instead, results from around the world have given even more comprehensive support to the Standard Model, with its three families of quarks and leptons interacting via the electroweak and quantum-chromodynamic forces. As a description of the available laboratory facts (see D. J. Miller *Nature* **349**, 379–387; 1991) the Standard Model is almost all we need, although many of its features remain arbitrary and it ignores gravity completely.

Last spring there were signs from CERN and Stanford that Z^0 bosons were decaying into pairs of beauty (equivalently, bottom) quarks at a significantly higher rate than the Standard Model predicted. But a number of the experimenters have now re-analysed their data, and Alain Blondel (Ecole Polytechnique, Paris) reported a new world average in good agreement with the predictions. An updated fit to the parameters of electroweak theory now needs only one free parameter, the mass of the Higgs boson. The top quark mass is measured at Fermilab to be $175 \pm 6 \text{ GeV } c^{-2}$, very close to the prediction from a similar fit made just before top was discovered, which then had two free parameters, the top and Higgs masses. The fit now requires the Higgs mass to be less than $680 \text{ GeV } c^{-2}$ with 95% confidence. This is well within the reach of CERN's next approved machine, the Large Hadron Collider.

This is all very reassuring for the Standard Model, but the properties of neutrinos undermine any illusion that we know the whole story. There are now three distinct sets of evidence that neutrinos of any one species — electron, muon or tau — might transform and oscillate back and forth into one or both of the other two species in flight. The first set comes from the LSND collaboration (H. White, Los Alamos). Their neutrino beam has more muon neutrinos than electron neutrinos, but they see more electron-like events in their detector than they expect.

The second comes from experiments that look for solar neutrinos with energies of a few electron volts: they see too few electron neutrino interactions. Yoshiaki Suzuki (Univ. Tokyo) reported the first

data from the Superkamiokande detector in Japan, which contains 50,000 tonnes of ultra-pure water viewed by an array of photomultiplier tubes. One month with the new detector has produced as many events as the preceding few years with the old Kamiokande experiment, and confirms that they see only 42% of the event rate predicted by the 'Standard Solar Model' (J. N. Bahcall, *Neutrino Astrophysics*, Cambridge Univ. Press; 1989). If the shortfall in the Kamiokande events is



Ratio of observed J/ψ production rate to the rate calculated assuming no quark–gluon plasma formation. The NA50 experiment claims to have reached a high enough energy density to form a quark–gluon plasma which ‘dissolves’ the J/ψ particles: as the path length through nuclear material increases, fewer particles emerge.

compared with the shortfalls observed using a chlorine target (in the Homestake mine) and gallium targets (the SAGE and GALLEX collaborations), there is no consistent explanation that does not involve neutrino oscillations.

The third set of evidence comes from neutrinos of about 1 GeV, which come from cosmic ray interactions in the atmosphere. Too many electron-like events are seen, compared with muon-like events.

Vladimir Smirnov (Moscow State Univ.) described a range of ingenious models that can accommodate any or all of these three sets of results by invoking at least two possible new effects, each of which goes far beyond the Standard Model. One of them, a sterile neutrino (which does not even interact by the electroweak force, only by gravity), gives an extra degree of freedom to the oscillation between species. The other involves rapid resonant oscillations between the species when they pass through matter of the right density and/or magnetization, which would be very important in supernovae and in other high-energy astrophysical systems, as well as in the Sun.

The first clear evidence for a spectacular new QCD effect came from an experi-

ment at CERN called NA50, in which a high-energy beam of lead nuclei hit a lead target — so more than 400 nucleons (protons and neutrons) are involved in each collision (P. Petiau, Ecole Polytechnique). QCD is the hardest part of the Standard Model to check, because the forces carried by gluons between the quarks are so much stronger than either electromagnetism or the weak interaction. As the interaction energy increases, the QCD force gets weaker. In high-energy collisions between nuclei, the energy density (effectively the local temperature) in the central region rises above 250 MeV per cubic femtometre, and a phase-change is predicted in which hadronic matter, with the quarks confined, changes into a quark–gluon plasma where the quarks move freely throughout the high-temperature zone.

The signature of the formation of plasma in NA50 is a sharp reduction in the observed number of decays of J/ψ particles for events where the collision between the nuclei is most violent. The J/ψ is a compact bound state of a charmed quark and a charmed antiquark, both of them heavy and hard to produce. If they come from the same point in a single proton–proton interaction there is a good chance they will stick together to make a J/ψ , but if they then have to travel through femtometres of quark–gluon plasma they are expected to drift apart. The J/ψ will effectively ‘dissolve’ in the plasma, as the new experiment claims to see.

QCD is hard to test whenever its coupling is strong, but because it gets weaker at high energies, simple, perturbative calculations can be made to predict the properties of hadron jets produced in all kinds of collider at tens or hundreds of GeV. An impressive range of data all fit the theory with a single value for the strength of the quark–gluon interaction (M. Schmelling, Univ. Heidelberg).

But there are still loose ends, especially when the structure of the proton (or of the photon) is probed very deeply. Experiments at HERA show that low-energy gluons inside the proton are much more numerous than had been expected by most theorists (H. Abramowicz, Univ. Tel Aviv), and extra gluons may also be needed at the highest energies, to explain the number of very high-energy jets seen in the CDF experiment at Fermilab (R. Brock, Univ. Michigan).

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Amyloid ox-tox transducers

Mark P. Mattson and Russell E. Rydel

ACCORDING to the 'amyloid theory', the factor that initiates and promotes Alzheimer's disease (AD) is amyloid- β peptide ($A\beta$)¹. The theory emerged and has been consolidated in the 1990s, and is supported by two main lines of evidence — the association of $A\beta$ with degenerating neurons in AD brain; and the linkage of mutations in the β -amyloid precursor protein to some inherited forms of AD, and demonstration that the mutations result in increased production of $A\beta$. $A\beta$ -induced oxidative stress and disruption of cellular ion homeostasis are implicated in the neurodegenerative process^{2,3}. The peptide also activates microglia, the resident immune effector cells of the brain, which are mobilized in AD and may play a central role in inflammatory processes associated with amyloid plaques⁴.

A missing link, however, is how $A\beta$ interacts with the cell surface to induce free-radical production. Two reports in this issue now identify mechanisms for such interactions that may explain how $A\beta$ damages neurons and vascular endothelial cells, and activates microglia. Yan *et al.*⁵ (page 685) provide compelling evidence that $A\beta$ interacts with a cell-surface receptor called RAGE (receptor for advanced glycation end products) in neurons, microglia and vascular endothelial cells. Their data also demonstrate that this interaction mediates cell adhesion to $A\beta$ and induction of oxidative stress in microglia.

Using a similar experimental approach, El Khoury *et al.*⁶ (page 716) show that the unrelated class A scavenger receptor (SR) mediates adhesion of microglia to $A\beta$ fibrils and leads to secretion of reactive oxygen species and cell immobilization. Taken together (see figure), the two studies suggest that RAGE and SR are key players in the neurodegenerative process in AD, and that these receptors are potential drug targets.

RAGE and SR are distinct gene products and share little, if any, sequence similarity. RAGE is a member of the immunoglobulin superfamily of cell-surface molecules and is closest in sequence to the neural cell-adhesion molecule; is expressed in a variety of cell-types, including endothelial cells, smooth muscle cells, mononuclear phagocytes, and neurons; and is thought to mediate pathophysiological responses in the vasculature when occupied by glycated ligands⁷. SR is a homotrimeric cell-surface molecule that contains α -helical coiled-coil, collagen-like and cysteine-rich domains; is expressed only on resident macrophages or microglial cells; and promotes the endo-

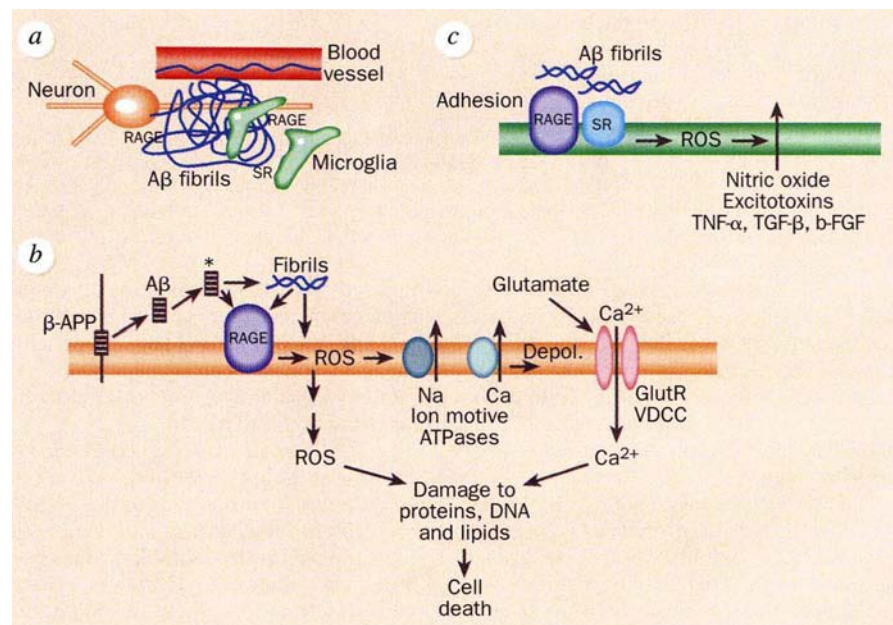
cytosis and degradation of modified low-density lipoproteins and glycated ligands⁸. However, both receptors are upregulated in affected regions in AD brain and are characterized by having a high affinity and broad specificity of ligand binding; in particular, both are bound by advanced glycation end products (AGEs)⁹.

$A\beta$ is normally produced in a soluble monomeric form that circulates at low levels in the cerebrospinal fluid and blood. To some extent in normal ageing, and much more in AD, it forms insoluble fibrillar aggregates. These aggregates are associated with degenerating neurons and activated microglia in AD brain, and cell-culture studies have shown that the process of fibril formation is tightly correlated with the ability of the peptide to damage neurons and activate microglia^{1,4}.

The new studies^{5,6} suggest that RAGE and SR are involved in signalling microglia to accumulate at sites of $A\beta$ deposition. They provide convincing evidence

that microglia adhere strongly to $A\beta$ fibrils, that $A\beta$ can induce microglial activation, and that both of these effects can be mediated by RAGE and SR. This molecular interaction may account for the well-documented inflammatory reactions that occur in AD brain. It has been assumed that the recruitment and activation of microglia in plaques is detrimental to neurons because activated microglia produce potentially neurotoxic substances including reactive oxygen species and excitotoxins⁴. But activated microglia also produce neuroprotective substances including transforming growth factor- β (TGF- β) and basic fibroblast growth factor (b-FGF). Yan *et al.*⁵ show that $A\beta$ induces production of tumour necrosis factor- α (TNF- α) by microglia, an effect blocked by anti-RAGE antibody. Although the conventional wisdom is that TNF- α is a 'bad guy', it can protect cultured neurons against $A\beta$ toxicity¹⁰, and studies of mice lacking TNF- α receptors suggest that microglial activation and neuronal damage may not be connected¹¹.

Yan and colleagues' data also implicate RAGE in the neurotoxic action of $A\beta$, although here the evidence is less com-



a, Neuritic plaques in AD brain are composed of fibrils of amyloid- β peptide ($A\beta$) that are in close apposition to degenerating neurons and activated microglia; $A\beta$ is also deposited in blood vessels. $A\beta$ binds to the receptor for advanced glycation end products (RAGE) on the surface of neurons, microglia and vascular endothelial cells, and to the scavenger receptor (SR) on microglia.

b, Mechanisms of interaction of $A\beta$ with neurons. Enzymatic processing of the β -amyloid precursor protein (β -APP) generates $A\beta$ which is released from brain cells in a soluble form. Under oxidizing conditions $A\beta$ may form free-radical moieties (*) and peptide fibrils. Interaction of $A\beta$ with the cell surface of neurons induces production of reactive oxygen species (ROS) in the cell membrane (lipid peroxidation); RAGE may mediate generation of ROS. The ROS impair the function of membrane proteins involved in maintenance of ion homeostasis resulting in membrane depolarization and calcium influx through glutamate receptor channels (GlutR) and voltage-dependent calcium channels (VDCC). ROS and calcium promote damage to cell proteins, DNA and lipids, and ultimately cause cell death.

c, $A\beta$ interaction with, and activation of, microglia. Binding of $A\beta$ fibrils to RAGE or the SR on the surface of microglia promotes cell adhesion, and induces activation of the microglia resulting in microglial generation of nitric oxide, excitotoxins, cytokines such as tumour necrosis factor- α (TNF- α) and transforming growth factor- β (TGF- β), and the neurotrophic factor basic fibroblast growth factor (b-FGF).

pellings. The approach for assessing neuron viability involved use of the dye MTT, which is an indicator of mitochondrial respiration rather than cell viability as such. Previous studies^{1,2} have shown that subtoxic levels of A β can induce a marked decrease in MTT reduction in neural cells similar to that observed by Yan *et al.* But although Yan *et al.* clearly show that RAGE induces production of reactive oxygen species in microglia, they do not show that RAGE mediates oxidant stress in neurons, and do not conclusively link oxidant stress with A β -induced cell death. So it remains to be seen whether RAGE does, in fact, mediate A β toxicity. RAGE selectively recognizes proteins to which sugar residues have been added by the process of glycation. Nevertheless, pure synthetic A β (in the absence of glycation) induces oxidative stress and is cytotoxic^{1,3}, bringing into question both the specificity of binding of A β to RAGE and the proposed role of A β glycation in inducing oxidant stress².

A possible explanation for A β binding to RAGE and SR comes from data showing that A β itself can generate free-radical peptide moieties¹³ which could mediate peptide crosslinking, cell adherence and neurotoxicity. Yan *et al.*⁵ demonstrate that cells expressing RAGE exhibit increased lipid peroxidation when exposed to A β compared to mock-transfected controls. But they do not establish that this peroxidation effect is specific for A β , raising the possibility that cells transfected with RAGE or SR are more sensitive to oxidative stressors (receptor- or nonreceptor-mediated) in general. Whether binding of A β to RAGE and induction of membrane-lipid peroxidation are directly linked is not known. It will be important to establish how binding of A β to RAGE or SR triggers oxyradical production. A signal-transduction pathway linked to RAGE or SR has not been identified, and these receptors are thought to function mainly as mediators of endocytosis or transcytosis of macromolecules.

An important effect of A β on neurons is that it can make them more vulnerable

to the metabolic, excitotoxic and oxidative stressors that accrue as the brain ages¹⁴. By inducing membrane-lipid peroxidation, A β can impair the function of membrane-regulatory proteins, including ion-motive ATPases; these actions of A β disrupt ion homeostasis and promote activation of N-methyl-D-aspartate (NMDA) receptors and sustained increases in levels of intracellular calcium which contribute to the cytotoxic action of A β (ref. 3). If RAGE mediates the oxidation-inducing effects of relatively low concentrations of A β , it may be particularly important in promoting excitotoxic cascades.

Such an 'endangering' action of A β -RAGE interaction could explain why some types of non-neuronal cells which do not express NMDA receptors (astrocytes and fibroblasts, for example) are resistant to being killed by A β . Interestingly, Yan *et al.* provide evidence that levels of RAGE are increased in AD brain, particularly in neurons associated with neuritic plaques;

levels of SR are also increased in AD brain⁹. Whether these increases precede the deposition of A β fibrils or follow cell damage remains to be determined.

It is likely that more cellular receptors that are directly or indirectly affected by A β will emerge; for example, there is already a report that the serpine-enzyme complex receptor recognizes soluble, non-toxic A β but not aggregated, cytotoxic A β (ref. 15). Nevertheless, the new data identify RAGE and SR as possible targets for the development of drugs designed to reduce the devastating neuronal injury, inflammation and vascular damage that occurs in the brains of AD victims. □

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PALAEOECOLOGY

Hunting ground for farmers

Peter D. Moore

THE landscape of northern Europe is so dominated by agricultural activity and its consequences that it is difficult to imagine the region before the arrival of the agricultural idea. Perhaps this is why we conceive the coming of agriculture as a cataclysmic event — a Neolithic revolution. But some of the most detailed palaeoecological analyses ever conducted in the British Isles, as a result of the work of Ian Simmons and John Innes published recently in the *Journal of Archaeological Science*¹, are telling a different story. Cereal cultivation in northern Britain came in with a crackle and pop rather than a bang: our daily bread, it seems, was first produced as a mere afterthought by our hunter-gatherer ancestors.

The first signs of farming in northwest Europe are detected in pollen diagrams of peat profiles and lake sediments by the incidence of cereal pollen, together with an increasing concentration of weed pollen, indicative of disturbed ground, and a reduction in tree pollen, showing the development of clearings in former closed-canopy woodland². Such identification of early farming is not always simple, partly because natural disturbance of forests by wind-throw or fire, or pre-agricultural activities on the part of our species, can create conditions that are essentially indistinguishable from those generated by the first farmers, and also because the pollen of the cereals, especially primitive varieties, are extremely difficult to separate with certainty from the pollen grains of certain wild grasses³.

This taxonomic problem is particularly acute when we are dealing with just a few isolated fossil grains resulting from small-scale agriculture⁴.

It has long been agreed that agriculture was practised by the Neolithic peoples inhabiting northern Europe at the time of the decline of the elm — a marked and roughly synchronous event occurring about 5,400–4,600 radiocarbon years before present and involving a decrease in elm pollen from around 15–20 per cent of the total pollen input to less than 5 per cent. Arguments concerning the human involvement in this event, possibly by selectively exploiting the elm as animal fodder, or perhaps aiding the spread of disease among elm populations, have long occupied the minds of palaeoecologists, and are still in dispute⁵. More recently, however, attention has centred on disturbance episodes before the elm decline, with the intention of sorting out the conditions under which cereal cultivation was first introduced.

Some of the most valuable information has emerged from work in the North York Moors of northeastern England, where blanket peats at an altitude of 350 metres contain evidence of disturbance episodes before the decline of elms, some with traces of cereal pollen. Turner, Innes and Simmons⁶ have used close-sampling techniques to elucidate in detail the changes taking place within these disturbances, sometimes resorting to the analysis of contiguous 1-mm slices of peat to maximize resolution. In their latest experi-

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Open evidence. In Broxa Forest on the North York Moors, G. W. Dimbleby established a set of plots where natural regeneration was allowed to occur. Young birch here surrounds an opening dominated by heather — like some of the later stages of prehistoric openings inferred from pollen analysis. (Photograph courtesy of I. G. Simmons.)

ments, Simmons and Innes¹ analysed 70 samples in this way, taking them through an episode in which the pollen of *Triticum* (wheat) occurs for the first time. Their temporal resolution is such that each sample is reckoned to comprise just six years' pollen rain, so the event recorded took about 400 years.

Before disturbance occurred, the forest consisted of deciduous trees (oak, alder, hazel and elm), with some birch and willow. These latter two trees, together with a range of herbs, show that the forest had some open (presumably natural) clearings. A more persistent and marked clearance is then shown by an abrupt fall in first alder and then oak pollen, and a general increase in indicators of open, disturbed conditions. Most marked among these indicators is *Melampyrum* (cow-wheat), which is a semi-parasitic plant often associated with clearings, trampled areas and sometimes burnt habitats. These conditions continue for some time (about 20 mm of peat depth, which is equivalent to about 120 years) before the first appearance of wheat pollen grains. With the appearance of cereal crops, the weed flora changes; plantain (*Plantago lanceolata*), mugwort (*Artemisia* sp.) and various species indicative of disturbed soil become more frequent. Evidently, the land management has changed at this stage and new management objectives have been adopted.

The authors regard the initial clearance and the maintenance of open conditions as a means of habitat management for the promotion of hunting activities. Red deer, a major prey species of Mesolithic cultures and a common animal in upland forests, was undoubtedly encouraged by the creation and maintenance of open, regenerating areas of forest. Hunting must also have been

facilitated by the clearance of glades within the forest. So we can imagine a population of hunters managing clearings by felling and fire for more than a century before the arrival in the region of the agricultural idea, together with the wheat grains that were ultimately to revolutionize the way of life of the people of the area. Simmons and Innes claim that "cereal growing was slipped into a patch of land which had hitherto been used for another purpose". Perhaps that former usage had even proved an effective preparatory stage for cereal cultivation, with fire and increased animal dung input enriching the constantly disturbed soils.

What is also apparent is that cereal growing was abandoned at the site after another fifty years or so, possibly as a result of soil exhaustion, or poor crop productivity at this altitude, or the sheer effort involved in protecting crops from the deer that the clearing had originally encouraged. Whatever the reason for the abandonment of the agricultural experiment, it is clear that, far from being a Neolithic revolution, early cereal growing in this part of Yorkshire was not only an afterthought, but was also a bit of a flop. □

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DAEDALUS

Clearing the air

Most domestic smoke alarms have a feeble radioactive source, the alpha-emitter ²⁴¹Am. The alpha particles ionize the air in the device, permitting a weak current to flow between two electrodes. The light ions carrying this current are readily captured by smoke grains or other fine particles in the air. Once stuck firmly onto large, heavy smoke grains, the ions travel much more slowly. The current falls, and an amplifier sounds the alarm.

Particles suspended in the air bring all sorts of trouble. Exhaust fumes cause grime and pollution; pollen, asbestos fibres, airborne viruses and bacteria spread ill health; and these days cigarette smoke is the most resented of them all. Can smoke-alarm technology, asks Daedalus, be adapted to remove them?

The principle seems simple enough. A suitable beam of alpha radiation should rapidly put a charge on every particle in the local air. Two large plates with a few thousand volts across them — in effect, a sort of radioactively augmented electrostatic precipitator — should then attract the charged grains from the air. Sadly, as Millikan found in his determination of *e*, charged droplets drift very slowly, even in strong fields.

So Daedalus is abandoning the charged plates. An alpha track must contain equal numbers of positive and negative ions. Of the grains that capture them, half will go positive while the other half will go negative. These will be mixed together at very small separations, and will attract each other forcefully. This strong local attraction, aided by brownian wandering, will rapidly bring the two types together. On impact, each pair will form a neutralized clump. Each clump will take up more charge, and clump again and again.

Daedalus concludes that a suitable alpha source must rapidly agglomerate dust particles in the air into very coarse grains. And coarse grains are not hazardous. Our noses filter them out very efficiently. Even asbestos fibres are only dangerous if they are very fine. In any case, a coarse air suspension must soon fall to the ground as ordinary dirt.

So DREADCO's 'Dust-Slayer' is simply a suitable highly ionizing but short-range alpha source, with a fan to blow the room air steadily past it, clumping the fine dust into a rapidly precipitated grit. To avoid endangering the customers, it will be carefully screened and shielded, and (like smoke alarms themselves) will mention its radioactive content only in very small print. Indeed, a combined Dust-Slayer and smoke alarm seems feasible. It will agglomerate all the smoke in the vicinity, allowing the victims to breathe it safely.

David Jones

Brain cannabinoids in chocolate

SIR — Chocolate craving, common in western societies, is still incompletely understood. Although sensory components of the nervous system are likely to be essential¹, the association of chocolate craving with certain drug-induced psychoses² suggests that pharmacologically active substances could also be involved. Attention in this respect has been focused primarily on the methylxanthines³, which are thought to act as competitive antagonists at adenosine receptors⁴. We report here on a novel group of pharmacological constituents of chocolate, whose main target may be the endogenous cannabinoid system of the brain.

Anandamide (*N*-arachidonylethanolamine) is a brain lipid that binds to cannabinoid receptors with high affinity and mimics the psychoactive effects of plant-derived cannabinoid drugs⁵. It is released from neurons⁶ and is rapidly broken down by a selective enzyme activity⁷, suggesting that it may be an

endogenous cannabinoid neurotransmitter or neuromodulator. We considered that chocolate, which is rich in fat, might contain lipids chemically and pharmacologically related to anandamide. To test this possibility, we subjected samples of cocoa powder or chocolate (50 mg), obtained from three manufacturers, to sequential fractionations by solvent extraction, column chromatography, high-performance liquid chromatography and gas chromatography/mass spectrometry (GC/MS). We isolated three compounds that eluted from the GC at the same retention times as anandamide, *N*-oleoylethanolamine and *N*-linoleoylethanolamine, and displayed electron-impact mass spectra characteristic of these *N*-acylethanolamines and identical to those of synthetic standards (Fig. 1, and data not shown). Among the samples we analysed, the concentration of total unsaturated *N*-acylethanolamines varies from about 0.5 to 90 $\mu\text{g g}^{-1}$. In most cases, the rank order is *N*-oleoylethanolamine > *N*-linoleoylethanolamine > anandamide = 0.05–57 $\mu\text{g g}^{-1}$. By contrast, we detected no unsaturated *N*-acylethanolamines in white chocolate (a milk- and cocoa-butter-containing sweet used as a control for chocolate in behavioural studies)¹ or in brewed espresso coffee (whose pharmacological effects are attributed to caffeine, another methylxanthine).

N-oleoylethanolamine and *N*-linoleoylethanolamine do not activate brain cannabinoid receptors and their biological actions have not been yet defined. We found that *N*-oleoylethanolamine and *N*-linoleoylethanolamine inhibit anandamide hydrolysis in rat brain microsomes, a reaction catalysed by anandamide amidohydrolase activity⁷ (Fig. 2a). Moreover, *N*-linoleoylethanolamine produces a similar inhibitory effect in intact cells. Rat cortical astrocytes in culture hydrolyse exogenous [³H]anandamide to ethanolamine and [³H]arachidonate. Arachidonate is readily incorporated into phosphatidylcholine and phosphatidylethanolamine⁶. In the presence of *N*-linoleoylethanolamine, degradation of [³H]anandamide by the astrocytes is strongly reduced, and the amount of residual [³H]anandamide increased correspondingly (Fig. 2b). The concentration of *N*-linoleoylethanolamine that inhibits [³H]anandamide hydrolysis by 50% is ~5 μM (Fig. 2b, inset).

Our results demonstrate that cocoa powder and chocolate contain three unsaturated *N*-acylethanolamines that could act as cannabinoid mimics either directly (by activating cannabinoid receptors) or indirectly (by increasing anandamide levels). Further experiments are necessary to determine whether the concentrations of unsaturated *N*-acylethanolamines measured in our study are sufficient to produce these biological effects *in vivo*.

How can activation of the endogenous cannabinoid system participate in the sub-

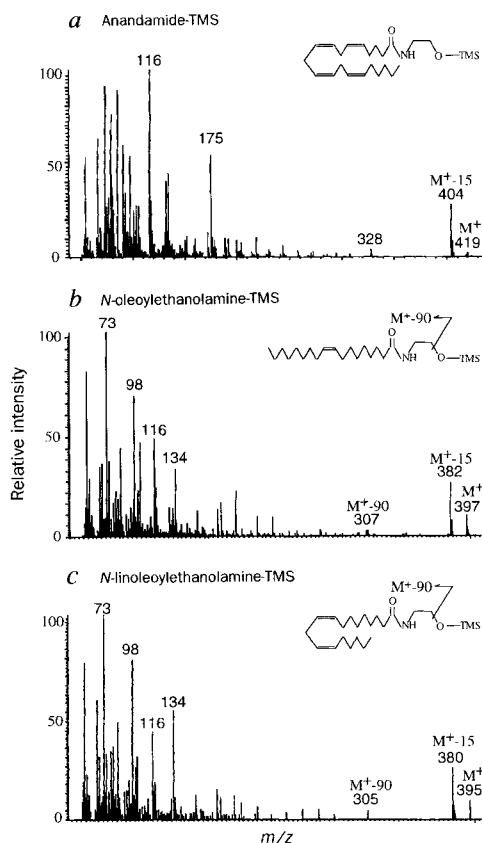
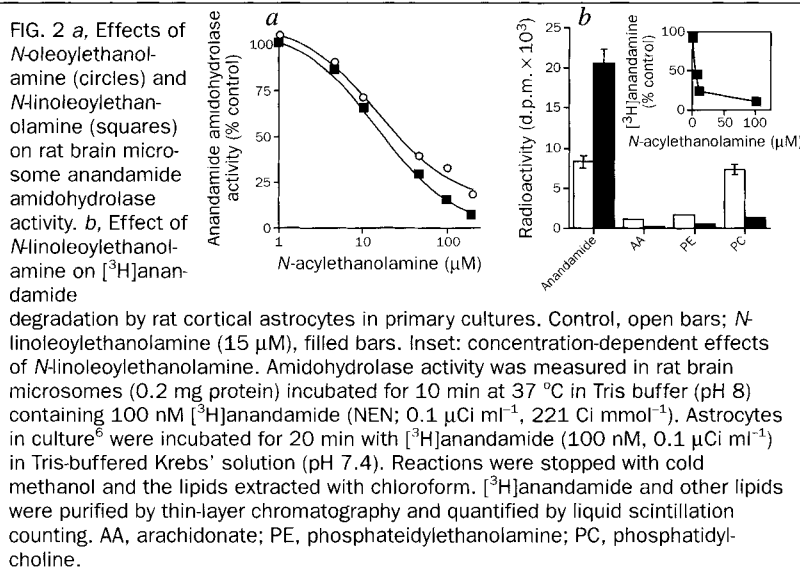


FIG. 1 Electron-impact mass spectra of the trimethylsilyl (TMS) ether derivatives of *a*, anandamide; *b*, *N*-oleoylethanolamine; *c*, *N*-linoleoylethanolamine, isolated from a 50-mg sample of commercial cocoa powder. The spectra are identical to those of synthetic standards (not shown).



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jective feelings associated with eating chocolate, and in chocolate craving? Cannabinoid drugs are known to heighten sensitivity and produce euphoria⁸. A possible effect of elevated brain anandamide levels could be to intensify the sensory properties of chocolate thought to be essential to craving. Alternatively, elevated anandamide levels could cooperate with other pharmacological components of chocolate (for example, caffeine, theobromine) to produce a transient feeling of

well-being. Whether or not any of these speculations turn out to be correct, our results point to an unexpected link between non-drug craving and the endogenous cannabinoid system, which deserves further examination.

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Transcriptional activation by BRCA1

SIR — Mutations in the gene *BRCA1* may account for as much as 90% of inherited breast and ovarian cancers in families predisposed to both maladies. *BRCA1* encodes an 1,863-amino-acid protein containing a putative zinc-ring finger domain¹, suggesting that *BRCA1* binds DNA. To date, no function has been ascribed to *BRCA1*. Here we report that the carboxy-terminal portion of *BRCA1* acts as a strong transcriptional transactivator when fused to the GAL4 DNA-binding domain and that this activity is completely abolished in sequences corresponding to four different mutations found in *BRCA1* families.

We inserted portions of the *BRCA1* complementary DNA into the plasmid pSG424 to create in-frame fusions with the GAL4 DNA-binding domain. We co-transfected the resulting plasmids into

293T cells, a human kidney-derived cell line², together with the reporter-gene plasmid pGAL4BCAT, which contains 5 GAL4 binding sites and a minimal promoter linked to the chloramphenicol acetyltransferase gene.

We detected no activity for the GAL4 DNA-binding domain (DBD) alone, nor when it is fused to amino acids 1–1,652 of *BRCA1*. Three central portions of *BRCA1* also fail to show activity (*a* in the figure). However, a C-terminal portion of *BRCA1* (amino acids 1,528–1,863) shows significant transcription activation of the reporter when fused to the GAL4 DBD, whereas a smaller C-terminal fragment (1,760–1,863) shows moderate activity. We conclude that amino acids 1,760–1,863 constitute a minimal region required for transactivation, and that additional sequences N-terminal to this region are

required for maximal activity.

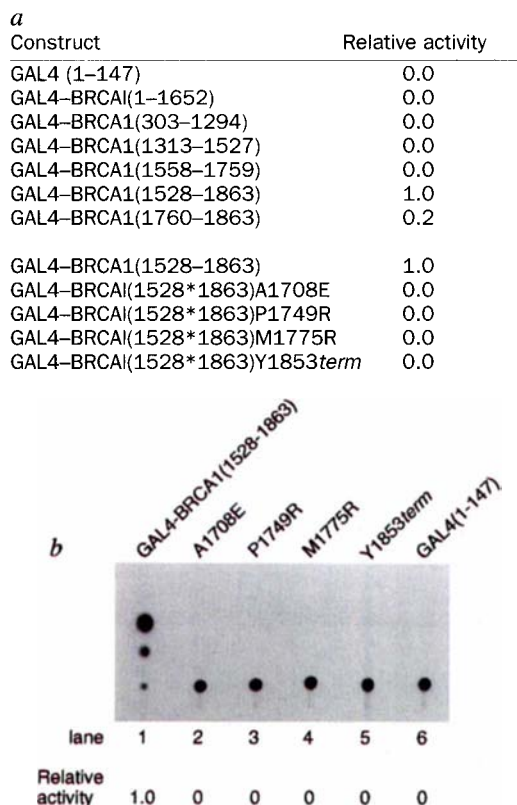
More than 134 distinct mutations in *BRCA1* have been identified among families with a *BRCA1*-linked predisposition for breast and ovarian cancers³. Most of these are frameshift mutations which result in a truncated *BRCA1* protein lacking C-terminal sequences. In addition, several *BRCA1* mutations have been described in which single amino-acid changes occur in the C-terminal portion of the protein.

To determine what effect such mutations might have on the transcriptional transactivation activity of *BRCA1*, we made GAL4-*BRCA1* fusion proteins corresponding to four C-terminal mutations (*b* in the figure). The *BRCA1* point mutants A1708E (where the alanine residue at position 1,708 is changed to glutamate) and M1775R (methionine to arginine) are almost certainly associated with a predisposition to breast and ovarian cancers^{1,3,4}, whereas the disease association of the mutant P1749R (proline to arginine) is less certain, occurring in a patient with ovarian cancer but not in controls⁵.

We also made a fourth mutant, Y1853term, in which the addition of a single nucleotide creates a termination codon at position 1,853, thereby deleting the final 11 amino acids of *BRCA1*. This *BRCA1* mutation has been found in several individuals with breast cancer, and is definitely associated with a predisposition to breast and ovarian cancer (ref. 6, and M.-C. King, personal communication). When each of these four *BRCA1* mutations is introduced into GAL4-*BRCA1*(1528–1863), we see no transactivation of the reporter gene (*b* in the figure), even when we used very high levels of the cell extract. In addition, when we introduced the mutation M1775R into the context of GAL4-*BRCA1*(1760–1863), this fusion construct showed about half the activity of its wild-type *BRCA1* counterpart, GAL4-*BRCA1*(1760–1863) (data not shown).

When using western analysis with a polyclonal anti-GAL4 DBD antibody, we find no large differences in steady-state protein levels between the wild-type or mutant proteins in the GAL4-*BRCA1*(1528–1863) context, although the levels of A1708E and Y1853term are somewhat reduced. Polyclonal anti-*BRCA1* antibody detects the identical bands when the membranes are reprobbed. Lack of transactivation by Y1853term mutant further defines the transactivation domain in *BRCA1*, in that the final 11 C-terminal amino acids are required for activity. It is somewhat surprising that mutants A1708E and P1749R show a complete lack of transactivation, because GAL4-*BRCA1*(1760–1863), which does not include these amino acids, still has some activity.

The involvement of *BRCA1* in familial
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a, Transactivation by the C terminus of *BRCA1*. We fused portions of the *BRCA1* coding region in-frame to amino acids 1–147 of GAL4 (ref. 10) to create the GAL4-*BRCA1* fusion proteins shown. We co-transfected fusion constructs with the reporter plasmid pGAL4BCAT (ref. 11) into 293T cells² and determined CAT activity at 24 h following transfection. Values represent at least three separate experiments. We included RSV- β gal plasmid for normalization of transfection efficiency. *b*, *BRCA1* mutants lack transactivation efficiency. Mutant *BRCA1* sequences were made and fused to the GAL4 DNA-binding domain. 293T cells were transiently transfected and CAT assays done as in *a*.

breast and ovarian cancer is well established, but the mechanism of tumour suppression by BRCA1 remains to be elucidated. Here we have defined a transcriptional transactivation activity by the C-terminal portion of BRCA1, a result consistent with the reported nuclear localization of BRCA1 (refs 7–9, and H. Ruffner and I. V., unpublished observations). Although some protein sequences fortuitously show transcriptional transactivation activity, the lack of this activity in four different *BRCA1* mutations in families predisposed to breast and ovarian cancer suggests that transactivation may be a true function of BRCA1. If so, we would expect this gene to bind DNA and to regulate a set of target genes. Demonstration of such activities is necessary to confirm our find-

ings and to improve our understanding of the role of *BRCA1* in tumour suppression.

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Insect vibrational defence signals

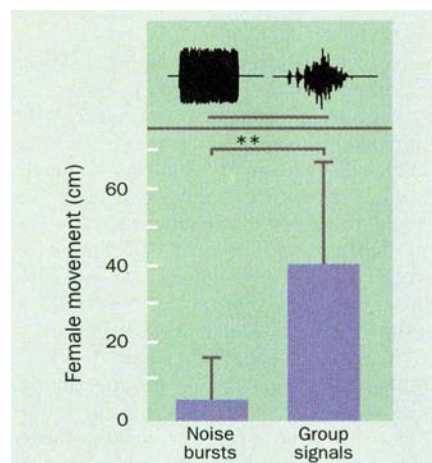
SIR — A hallmark of insect eusociality is the existence of elaborate communication systems among colony members that permit adaptive responses to changes in the environment¹. I describe an analogous communication system in a subsocial insect, the treehopper *Umberia crassicornis* (Insecta: Homoptera: Membracidae). In this species, females provide care for aggregations of nymphal offspring². Aggregations extend for up to 15 cm along the host-plant stem and contain up to 80 individuals. When attacked by a predator, nymphs produce substrate-borne vibrational signals that rapidly elicit their mother's antipredator behaviour.

Signals produced by nymphs at the site of the attack prompt signalling by their siblings. The result is a composite, group signal, and only group signals evoke the mother's antipredator response.

Nymphal *U. crassicornis* are preyed upon by a range of invertebrates^{3,4}. Their most important predators are wasps, which include treehopper specialists^{5,6}. Maternal protection, including approaching the predator, wing fanning and kicking with club-shaped hind legs^{3,6}, is the nymphs' only defence against attack by invertebrate predators.

The vibrational signal produced by a nymph is a brief series of pulses lasting

Responses of female *U. crassicornis* to vibrational playback (representative playback stimuli are shown in upper panel, where scale bar represents 1 s). In response to group signals, previously stationary females rapidly approached the area where the aggregation had been, then engaged in apparent searching behaviour; in response to noise bursts (which differed from natural signals in the time and frequency domains), most females remained stationary (Wilcoxon signed-rank test, $n=14$, $P < 0.01$). The measure shown represents distance moved by females during a 3-min stimulus period. Signals contained energy mainly from 100–4,000 Hz and were transduced with a lightweight accelerometer (0.28 g; frequency response flat from 20–5,500 Hz) glued to the woody host-plant stem. Stimuli were played from a Macintosh IIsx computer and coupled to the host-plant stem using an electrodynamic shaker (Labworks ET-203). As found in other studies⁷, the resulting signals propagated in the plant stem were bending waves; this was also true of the natural signals (my unpublished data). The vibrational playback system used a closed-loop procedure to ensure that playback signals matched natural signals in amplitude and frequency characteristics. Shaker output was monitored with an accelerometer at the site of the playback subject to calculate the filtering characteristics of the entire system. Inverted filter coefficients were derived to compensate for this filtering (which was largely due to the plant transmission channel), and these were applied to the playback stimuli. As a result, when a natural signal was played back, it closely matched the frequency spectrum of that same signal when first recorded at that site (amplitude spectra equal to within 0.5 dB in 164-Hz bins up to 5.5 kHz). Stimulus pairs were adjusted to be equal in peak amplitude to natural signals, and to each other, at the site of the playback subject. The order of stimulus presentation was alternated between subjects in each experiment.



30–40 ms. After one individual is attacked by a predator, signalling travels in a wave through the aggregation. Individual signals thereby combine to form a composite, group signal with a duration of 300–600 ms. Disturbed aggregations produce group signals at intervals of 1–2 s.

Females respond to vibrational playback of group signals, but not simply to any vibrational stimulus from the aggregation site. Females rapidly approached the vibration source in response to playback of group signals from their offspring (removed from the plant during playback), occasionally kicking and wing fanning. In contrast, most females remained stationary (as during prestimulus periods) in response to noise bursts of the same duration, repetition rate, and peak amplitude (see figure).

Coordination among signalling nymphs is necessary to evoke the female's response. Playback of 10 individual offspring signals digitally combined into the coordinated pattern of natural group signals caused females rapidly to approach the signal source, as in the previous experiment. However, females showed little or no response to the same signals arranged in random temporal patterns (Wilcoxon signed-rank test, $n=12$, $P < 0.01$).

How is this coordination achieved, given that only one or a few nymphs may be contacted directly by a predator? Playback of group signals (with the female removed from the plant) consistently elicited signalling from otherwise undisturbed nymphs, while playback of noise bursts of the same duration, peak amplitude and repetition rate did not (Wilcoxon signed-rank test, $n=15$ aggregations, $P < 0.01$). Even when they are not immediately threatened, nymphs will thus signal in response to signals from siblings.

During field observations of the closely related species *U. spinosa*, group signalling occurred almost exclusively in the presence of predators. In 7 days of observation of 10 aggregations near Gamboa, Panama, I observed 60 predation attempts on nymphs by vespine wasps (primarily *Pseudopolybia compressa*). Undisturbed nymphs were silent or produced sporadic individual signals, but nymphs attacked by wasps produced a rapid series of vibrational group signals. Females successfully defended nymphal aggregations in 54 out of 60 attacks (90%), and in only 6 cases (10%) were

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wasps successful in removing a nymph. In contrast, the success rate of predatory wasps can be as high as 90% in the absence of tending females⁶. Offspring-parent signalling appears to play a central role in defence in these subsocial insects. As in eusocial taxa, communication among group members permits an adaptive response to a rapidly changing feature of the environment.

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Classic clues to NSF function

SIR — Most intracellular membrane fusion events require the action of the *N*-ethylmaleimide-sensitive fusion protein (NSF)¹. The role of NSF in vesicular transport remains highly controversial, and various models have proposed that NSF acts at a post-docking stage close to membrane fusion², at a post-docking but pre-fusion stage³, or at a pre-docking stage^{4,5}. A recent study of *in vitro* vacuolar fusion in yeast⁶ supports the latter hypothesis, as does re-examination of a 1976 report on the temperature-sensitive *Drosophila* mutant *comatose*⁷, which is now known to be an NSF mutant⁸.

Like many other *in vitro* membrane fusion assays¹, homotypic fusion of yeast vacuolar vesicles requires NSF (Sec18p in yeast). Using an elegant approach, Mayer *et al.*⁶ have succeeded in kinetically defining the stage of action of NSF in the com-

plex process of priming, docking and fusion of vesicles that comprises the vacuolar fusion assay. They found that the action of Sec18p is complete even before docking can occur, implying that NSF acts to prime vesicles for subsequent docking and/or fusion.

Although the literature is replete with data on NSF function in constitutive membrane traffic¹ such as the vacuolar fusion assay, the only functional evidence for a role of NSF in regulated membrane traffic, as reported in *Nature*⁸, is that the temperature-sensitive paralysis exhibited by *comatose* mutants of *Drosophila* are due to point mutations in the *dNSF-1* gene. The original, classic work on *comatose* by Siddiqi and Benzer⁷, when reinterpreted 20 years later, provides important information on the molecular function of NSF in neurotransmission *in vivo*.

Siddiqi and Benzer⁷ observed the kinetics of onset and recovery from temperature-induced paralysis in three *Drosophila* mutants: *para* (paralysed), *shi* (shibire) and *com* (comatose), all of which result from a presynaptic block of neurotransmission. *Para* mutants became paralysed within seconds of a temperature shift and recovered almost instantaneously, whereas *com* mutants required a minute to become fully paralysed and 30 minutes to recover; *shi* mutants were intermediate, displaying complete paralysis at 30 seconds and recovering after 20 minutes. Although phenomenological at the time, these data have profound functional significance as it is now known that the *para* gene codes for a voltage-dependent sodium channel⁹, the *shi* gene for dynamin¹⁰ and the *com* gene for NSF⁸.

Neurotransmission begins with an action potential and results in the release by exocytosis of neurotransmitter, which then signals to the postsynaptic cell (stages 1 and 2 in the figure). Nevertheless, this complex process can take place in less than 200 microseconds. However, the depletion of synaptic vesicles by exocytosis has to be balanced by replenishment of new synaptic vesicles via endocytosis in a process estimated to require many seconds¹¹ (stages 3–6 in the figure). The kinetics of temperature-induced paralysis fit this model, as *para* flies exhibiting defects in voltage-dependent Na⁺ channels (required to generate an action potential) recover from paralysis almost instantaneously, as would be predicted if an essential switch for neurotransmission was suddenly turned on. Indeed, such fast kinetics are exhibited not only by different allelic mutants of *para*, but also by *nap* (no action potential) and *tip-E* (temperature-induced paralysis-E) *Drosophila* mutants, which have different Na⁺ channel defects¹². In contrast, *shi* flies exhibiting mutations in dynamin (required for endocytic vesicle forma-

tion) take 20 minutes to recover fully from paralysis, as would be predicted if an essential switch for synaptic vesicle replenishment was suddenly turned on (the synaptic vesicle pool in *shi* mutants is replenished after 15 minutes¹³).

As the time taken for *com* flies to recover from paralysis is even longer than that required for *shi* flies and as the known allelic mutants of *com* display similar kinetics of paralysis, this suggests that the action of NSF is slower than the process of synaptic vesicle recycling. The new work on vacuolar fusion⁶ suggests that this action is the priming of vesicles for cell membrane docking and fusion. Synaptic vesicle recycling is thought to involve two processes, endocytosis and vesicle re-priming, both requiring many seconds¹¹. The slow kinetics of *shi* and *com* mutants⁷ support a model in which dynamin acts in endocytosis and NSF in re-priming recycled synaptic vesicles (see figure). Such a slow, priming action of NSF is consistent with the hypothesis that NSF acts as a molecular chaperone to fold *Botulinum* neurotoxin substrates into a conformation competent for vesicle docking and/or fusion⁴. In addition to the *Drosophila* mutants discussed above, there are many temperature-sensitive paralytic mutants with differing kinetics whose mutant genes are unknown¹². On identification of these genes, a rich source of data from previous decades should lead to insights on their *in vivo* molecular function in synaptic vesicle dynamics based on the kinetics of their paralytic phenotypes.

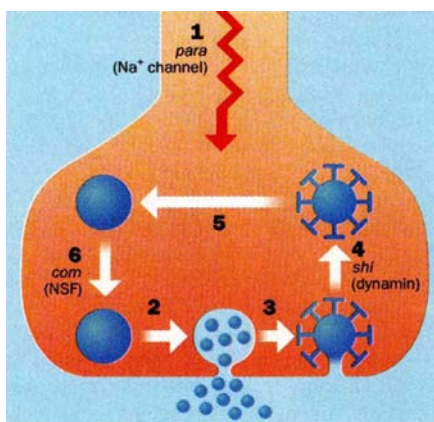
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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references.



Neurotransmission and synaptic vesicle recycling in *Drosophila* synapses. Stages depicted: 1, action potential propagation; 2, synaptic vesicle exocytosis and neurotransmitter release; 3, coated pit formation; 4, endocytic vesicle formation; 5, vesicle uncoating and reloading with neurotransmitter; 6, priming of vesicle for docking and/or fusion. The putative stages at which paralytic mutants are blocked in this cycle are illustrated.

Surfing with the aliens

Henry Gee

Independence Day. A Roland Emmerich film. Twentieth Century Fox: 1996.

MANY of the household names in science fiction cut their teeth in the pulp magazines that flourished in the 1930s and 1940s, immediately before the 'Golden Age' of science-fiction fandom. Back then, pulp science-fiction stories were often no more than Westerns set in space, and appealed to an audience of teenage boys (many of whom became well-known authors in their own right). As a genre, science fiction has moved on, propelled by an increasing literary sensibility. Sadly, many film-makers (and most literary critics) have missed this, and disparage science fiction as juvenilia. This treatment is no longer warranted, and the popularity of films such as *Independence Day* won't help.

Here's the plot. On 2 July of a year in the near future, enormous spacecraft appear over the Earth's major cities. (By 'the Earth', I mean, of course, 'the United States', with exceptions.) The spacecraft destroy the cities systematically, starting with Los Angeles, demonstrating that whatever their other failings, aliens have a keen sense of the aesthetic value of proper town-planning. The beleaguered US president (Bill Pullman), a former fighter-pilot, learns that the famous Roswell flying saucer recovered in New Mexico, and supposedly hushed up by the government, really exists. He gets to meet the aliens face to face, revealing them to be interstellar asset-strippers, interested only in plunder. The president and his bedraggled entourage use the Roswell flying saucer to spearhead a counter-attack against the aliens' mothership. The rest of the strike-force consist of anyone left in the world capable of flying a plane. Just two days later, on 4 July, humanity wins. The immense technology of intellects vast, cool and unsympathetic is soundly squashed by motherhood and apple pie. It's pure pulp, the only concession to the 1990s being that the two intrepid pilots of the requisitioned saucer aren't All-American WASPs, but a Jew from New York (Jeff Goldblum) and a black from Los Angeles (Will Smith).

The stars in this old-fashioned romp are the special effects, which are fantastic, and the film is enjoyable enough provided that one checks in one's brain at the cinema door. The trouble is that the plot hangs on several key improbabilities that one is asked to accept at face value.

How is it, for example, that Goldblum and Smith can fly the product of an alien technology with no more trouble than if it

were an unfamiliar rental car? How, exactly, can one disable the force-field screens of an alien spacecraft by 'infecting' it with a computer virus? How is it that Goldblum (a humble television technician) discovers this simple trick, when a team of dedicated government scientists had somehow overlooked it for 40 years? And how did the courageous mother

transcendent Overmind, a fate which it is their own tragedy not to share. Where is the film version of this mind-boggling tale? Again, one is reminded (inevitably) of *The War of the Worlds*. How much more satisfying it is for humanity to be helpless before the marauding Martians, who are destroyed by earthly bacteria rather than an addled old crop-duster pilot (Randy Quaid) let loose in an F-18.

Of course, the concept of huge alien spaceships was lampooned by Douglas Adams in *The Hitch-Hiker's Guide to the Galaxy*, in which the members of the alien fleet seem to hang in the air in the same way "that brick's don't". The fleet belongs to the Vogons, a heartless race charged

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

A scene from every Montana survivalist's dreams.

(Vivica Fox) manage a trek through the smoking ruins of Los Angeles without even once chipping her nail polish?

In *Jurassic Park*, at least, the science is explained just enough to give one a sense that events are controlled by a technology tantalizingly just beyond one's grasp. This is part of the fun, of course, and aids the suspension of disbelief necessary for any science-fiction story (or, indeed, any kind of story) to work.

Independence Day seems familiar because it wears borrowed clothes. It plays like one of those ensemble 'disaster' movies of the 1970s, such as *Towering Inferno* or *Poseidon Adventure*. The aliens come from the standard 'squishy horrible aliens' cupboard already much used by Ridley Scott in *Alien*. And there are playful references to *The Day the Earth Stood Still* and Arthur C. Clarke and Stanley Kubrick's film *2001: A Space Odyssey*.

While watching *Independence Day*, I was reminded of another Clarke story, published in 1954. *Childhood's End* begins in much the same way, with alien spacecraft hovering over the world's cities. After that, it's completely different. The aliens, complex characters in themselves, have the interests of humanity at heart, but they are not omnipotent. Their task is to shepherd humanity to a point at which it can join the

with demolishing the Earth to make way for an intergalactic freeway.

As Brian Aldiss and David Wingrove point out in *Trillion Year Spree*, a history of science fiction, it is a shame that most film-makers seem capable only of producing worn-out pulp re-treads such as *Independence Day* when there is so much that is good and filmable in the science-fiction canon. Where is the film of Walter Miller's *A Canticle for Leibowitz* or Olaf Stapledon's *Star Maker*? If modern authors such as Stephen King reach the screen, what about Iain M. Banks's *Consider Phlebas* or Greg Bear's *Eon*? Now, there's space opera for you.

There are plenty of exceptions, of course, such as *Blade Runner* and *Total Recall* (borrowings from Philip K. Dick) as well as *2001*. Perhaps most famously, *Forbidden Planet* is an unearthly reading of *The Tempest*. But *Independence Day* exists in a cosy pre-Golden-Age world of Hugo Gernsback pulp magazines, comfort-viewing for the millions who believe that flying saucers really exist, Elvis was abducted by aliens and the *X Files* is a documentary series. If so, *Independence Day* is pandering to the lowest common denominator, and its success suggests that we get the films we deserve. □

Henry Gee is an assistant editor of *Nature*.

Desirable biology

Dick F. Swaab

Queer Science: The Use and Abuse of Research into Homosexuality. By Simon LeVay. MIT Press: 1996. Pp. 364. \$33.50, £16.95.

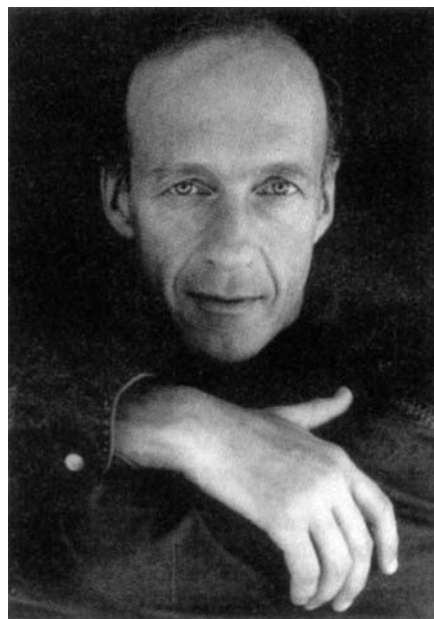
SIMON LeVay, a neuroscientist who left the Salk Institute in La Jolla, California, to co-found the Institute of Gay and Lesbian Education, has written an absorbing book on two important questions. What causes a person to be gay, straight or bisexual? And who cares?

A century after the German sexologist Magnus Hirschfeld formulated the first of these questions in *Sappho und Socrates* and Oscar Wilde served his prison sentence for committing several acts of "gross indecency", LeVay offers readers his personal views as well as a well-documented historical perspective on sexual orientation. It becomes clear how remarkably little change there has been in the various concepts of sexual orientation in medicine, biology, sociology, law and religion. It is good also to be reminded that it was not until 1973 that homosexuality was removed from the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders*, and, as a result, "20 million homosexuals gained immediate cure".

It is even more surprising to learn that even today people who believe that homosexuality is a matter of choice have a more negative attitude towards gays and lesbians than people who believe that homosexuals are 'born that way'. In 1992, Dan Quayle, then US vice-president, summed up his thoughts about homosexual men in the following way: "my viewpoint is that it's more of a choice than a biological situation... it's a wrong choice". Fortunately, discrimination and prejudice do not seem to be entirely 'inborn' or 'fixed'. By reading about the possible biological basis of homosexuality, students have been found to develop a less unfavourable attitude towards it. And this is why LeVay insists that research into homosexuality is worth pursuing, not only as a fascinating scientific topic, but also because it might help to change society's

attitude towards gays and lesbians. With the recent rapid progress in biological research on homosexuality, one can but hope that LeVay is right about biological theories of sexual orientation being "good for gay rights" and environmental theories being bad for them. In my own experience, feelings on the subject differ from country to country: when our group found the first gay-straight difference in the human brain in 1990, I was attacked, in 'progressive' Amsterdam, by some members of the Dutch gay movement who saw biological research into sexual orientation as a threat.

LeVay's criticism of Freud's convoluted explanations of homosexuality and of the



Simon LeVay: proponent of 'sexatypicality'.

theoretical justifications of attempts to 'cure' homosexuality — by transplanting testicles, by psychoanalysis, by inducing epileptic fits or by hormone treatment — are marvellously written and often refreshing. One of the stories LeVay tells is of the US Army unit that 'treated' 300 homosexual soldiers with testosterone during the Second World War, only to find itself in one of the worst homosexual situations imaginable: although increasing the soldiers' sexual drive, the treatment did not change their sexual orientation.

LeVay goes on to explain current ideas about the interaction of genes, sex hormones, aromatase (a brain enzyme that converts testosterone into oestrogens), environmental factors such as stress and the developing brain. This complex interaction is supposed to result in sex differences in brain and behaviour not only in animals but in humans as well. It would be worth seeing the faces of those who maintain that animals do not engage in same-sex behaviour when they read about 'homosexual' rams or the 'lesbian' seagulls in California.

Unfortunately, as soon as LeVay starts talking about his own field of research — sex differences in the human brain in relation to sexual orientation — his sense of perspective and his sense of humour disappear, and his ever-serious but playful writing changes. Suddenly he chooses to present only those data that fit one particular hypothesis of sexual differentiation, and he sacrifices the catholic style of the book for a particular concept of homosexuality, which he calls 'sexatypicality' and which presumes a more female brain in male homosexuals. This is basically the same idea as Gunther Dörner's brain-pseudo-hermaphroditism theory. At the same time, for reasons that never become clear, he strongly objects to the 'third sex concept': that is, the idea that the brains of homosexuals might, at least partly, be unlike those of heterosexual men or women. This leads to, among other things, his amazing statement that "[s]ince I (LeVay, 1991) published my findings on INAH-3 [a hypothalamic cell group], Laura Allen and her colleagues reported on a *second* difference between the brains of gay and heterosexual men (1992)" (my italics). In 1990, however, Michel Hofman and I described a difference in the suprachiasmatic nucleus between the brains of gay and straight men. That LeVay does not mention our findings smacks of his making a false claim of priority, although he has explained to me that this was unintentional. His reason for not mentioning our work, he tells me, was "that it did not fit the train of thought... since we [Hofman and Swaab] did not report that this difference was a sexatypicality".

Leaving aside for a moment the fact that it is good practice in science to adjust one's concept if the data do not fit it and not the other way around, and the fact that the suprachiasmatic nucleus is affected by sex hormones in development, I think it is far too early to embrace so definitely any one concept of sexual differentiation. The data on brain differences are so far much too fragmentary and controversial and first need replication and extension. There are indeed some observations, including LeVay's own, that fit the sexatypicality concept, but there are at least as many data that fit the 'third sex concept', or both. As usual in the field of neuroscience, it is hard to get at the truth; and LeVay is perfectly capable of explaining this difficulty to the public if he so wishes. One can only hope that in his next book he will succeed in putting this particular topic into perspective in the same stimulating style that characterizes his other chapters.

The last part of the book, on "Science Fiction—Science Future", is as compelling as the first part. Here LeVay deals with questions such as "Are we heading toward the day when a person's sexual orientation

■ Also just published is *A Separate Creation* by Chandler Burr, a journalist's smoothly written report on the frontlines of research into a possible genetic basis for homosexuality, and the moral and legal implications that follow. One of the more eccentric claims the author examines is that homosexuality is a genetic-bacterial condition that could be 'cured' with antibiotics. Hyperion/Bantam, \$24.95, £16.99.

will be covered by a simple blood test, brain scan or fingerprint analysis?", "Will it be possible to predict the future sexual orientation of a child or even of a fetus?", "Will the technology become available that can change a person's sexual orientation, or future orientation, by some form of brain engineering or genetic manipulation?" and "If any of these developments do become reality, will they be used for good or ill?". LeVay is not afraid to take a provocative stand. He does not believe that there should be legal prohibition of the use of genetic or neurosurgical techniques to alter sexual orientation, were such technology to become available. His guiding principle is simple: everyone has the right to life, liberty and the pursuit of happiness, so long as they do not infringe the rights of others. To clarify his position he draws a parallel with sex-reassignment surgery. An important question is whether science and society have procedures in place to minimize the dangers of such a liberal guiding principle. Good books such as this might certainly help — provided that we can find a way to persuade people who are not gay-friendly to read them. □

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When botany became no work for a lady

Londa Schiebinger

Cultivating Women, Cultivating Science: Flora's Daughters and Botany in England 1760 to 1860. By Ann B. Shteir. Johns Hopkins University Press: 1996. Pp. 301. \$29.95, £22.50.

HISTORIANS have tantalized us with the notion that botany was for a time considered a 'feminine' science. Here, finally, the Canadian historian of science Ann Shteir tells in detail how women 'elbowed' their way into botany at the end of the eighteenth century only to be 'elbowed out' again by the mid-nineteenth century.

According to Shteir, women botanists flourished in England from 1760 until 1830. How was this possible? As with many of the sciences in this period, botany was a widely appreciated amateur pursuit, fashionable among cultivated ladies and gentlemen. As long as botany remained an enriching pastime, women were welcomed as fellow enthusiasts.

In addition, botany posed no threat to orthodox views on women's nature: a rose was said to mirror the beauty of its feminine devotee; exotic plants were said to



A RARE blue iceberg lying just off Coronation Island, one of the South Orkney Islands. In *Antarctica*, Mike Lucas provides an authoritative account, accompanied by 300 colour photographs, of this continent's scientific, economic and ecological importance. New Holland, £29.99.

prefer delicate female nurturing; the female mind and body were encouraged to thrive on the rational pleasures botany afforded. Plants had long belonged to women's domains: peasants and aristocrats alike had worked as healers and wise women, gathering and cultivating the plants required for domestic medicines.

For a variety of reasons, then, by the end of the eighteenth century, botany among all the sciences was, according to the French philosopher Jean-Jacques Rousseau, considered least offensive to women. Botanists revelled in nature's verdant glory, while anatomists faced oozing blood and stinking cadavers, geologists dirt and filth, and entomologists their vile insects.

All this was to change as the amateur botanophile was replaced by the professional botanist. Shteir tells how, between 1830 and 1860 in England, the aristocratic ideal of polite knowledge embellishing an aesthetic, moral and spiritual sense of personal well-being gave way to a utilitarian ethic of practically useful science; how observational and field traditions in natural history succumbed to laboratory-based research; and how even textbooks turned from formats favouring lively and personalized conversations to those more formal and technical in tone.

As in other fields, professionalism was accompanied by a strident 'defeminization'. John Lindley, the first professor of botany at the University of London, declared: "It has been very much the fash-

ion of late years to undervalue the importance of this science, and to consider it an amusement for ladies rather than an occupation for the serious thoughts of man."

Shteir mentions several times that botany at the turn of the nineteenth-century was "dominated" by women. When women become prominent in surprising places, it is tempting to see them as dominant. But, according to Shteir's own account, women botanists were restricted in their activities — even at the height of their influence.

Lady Charlotte Canning, for example, served as an active collector from as far away as India. Had she been a man she might well have gone on a grand expedition in search of exotic specimens. As it was, she collected as a sideline to her main occupation, that of colonial wife, travelling where her husband happened to take her.

Women did, however, serve as influential patrons. Margaret Bentinck, the Duchess of Portland, commissioned naturalists to send her specimens from around the globe to add to her massive collection at Bulstrode Park. Women also served as illustrators, popularizers and educators. Priscilla Wakefield's *Introduction to Botany* (1796), for example, led the field for two generations.

Women's work only occasionally reached learned journals. Carl Linnaeus's daughter, Elisabeth Christina, published her observations on the phosphorescent effect on nasturtiums in the *Transactions of the Royal Swedish Academy of Sciences*.

Except for the field of herbal medicine, women by and large contributed to the diffusion of botanical knowledge rather than to its creation. They did not devise taxonomies, classifying the bounty of nature, nor did they set the agenda for botanical sciences, establishing for future generations what was to be known and not known about the vegetable kingdom.

The story of 'feminine' botany has had its focus in England. Because Shteir's volume again highlights England, we still do not know whether this is a uniquely British phenomenon or characteristic of botany throughout Europe, the Americas and other parts of the world. Natural theology, the attempt to derive the existence of God from empirical facts, was especially strong in England, which perhaps strengthened the appeal of botany to women there.

Shteir discusses in depth the many aspects of women's work in mainstream botany. But she does not discuss conflicts between what came to be recognized as

mainstream botany and women's traditional areas of knowledge, such as herbal medicines. The rise of male experts among midwives, later called gynaecologists, sounded the death-knell of knowledge on contraceptives and abortifacients (mostly herbal in nature) — knowledge traditionally belonging to women.

When women were moved out of botany, the consequences went well beyond the loss of livelihoods or opportunities — entire areas of expertise were lost or submerged.

In *Cultivating Women, Cultivating Science*, Shteir weaves intriguing biographies of women botanists into her intricate account of Victorian culture, science and society. This elegant book is essential reading for anyone interested in plants and science. □

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Imitating nature's designs

Robert W. Cahn

Biomimetic Materials Chemistry. Edited by Stephen Mann. VCH: 1996. Pp. 383. DM 189, \$125, £76.

'BIOMIMETIC' is a new word that has not yet made it into the *Oxford English Dictionary*. It refers to a novel concept, essentially a creation of the 1990s: the imitation of natural design. 'Biosynthesis' is a related term, meaning the imitation of chemical processes found in nature in order to synthesize materials that may not themselves be found in nature. In 1993, Paul Calvert, one of the contributors to this volume, listed some reasons, as they appeared then, for focusing researchers' interest on biological routes to polymeric materials: possible future oil shortages; promoting fermentation methods for making plastics and fibres from starch-derived feedstocks; increased demand for biodegradable plastics; and the rapid development of genetic engineering, permitting synthesis of proteins (and perhaps polysaccharides) by bacterial routes. He went on to cite some reasons for looking at natural biomaterials as inspiration for solving problems relating to load-bearing composites and ceramics. Since Calvert compiled his list, several journals in this field have been launched.

It is important to make distinctions. There is a thriving branch of science-based engineering devoted to the design of materials for medical purposes, such as bone prostheses, heart valves, replacement eye lenses and artificial blood vessels. As I write this, the proceedings

of the Sixth International Symposium on Bioceramics (that is, ceramics for medicine, as distinct from ceramics made by biological organisms) have just been published. So medical engineering is already a mature field. In the book reviewed here, however, the emphasis is on ways of making, or designing, materials by approaches borrowed from the biological world. Most of these materials are not intended for medical applications. Both biosynthesis and biomimetics form the subject-matter of the book, although only the latter term appears in the title. This complex of approaches derives from much earlier research on biochemistry and on the mechanical properties of natural materials, especially fibres, tendons, bone and exoskeletons.

The book's 22 authors contribute 13 chapters, most of which fall neatly into two classes. There are four chapters on the biosynthesis and biomimetics of ceramics and organic-ceramic composites. They are all written by people who would be content to be described as materials scientists, and there is a strong emphasis on achieving products with desirable properties, especially mechanical strength and toughness: these chapters are primarily about biomimetics. Six chapters have a much more strongly chemical flavour and concentrate more on biosynthesis than biomimetics. They include topics such as template-directed nucleation of inorganic materials, 'biogenic' cadmium sulphide semiconductors (by way of phytochelatins) and those fashionable entities, nanostructures. The two groups

of papers mostly pursue different approaches, but there are certain areas of overlap, notably the topic of inorganic structural materials in organisms, single-crystal exoskeletons in particular, and a focus on the mechanisms by which, for instance, single-crystal exoskeletons (as in some diatoms) can come into existence.

Finally, there are three unclassifiable chapters: one a fascinating chapter on using the venerable Langmuir-Blodgett technique to make organized films of minute particles (two-dimensional crystals, as it were) by flotation on a liquid surface and then transferring these to a solid substrate; another, an extraordinary account of bacterial fibres and mineralized products made from them. It seems that bacterial cells under certain circumstances can aggregate one-dimensionally to form 'ropes' of considerable strength. These ropes are solutions still looking for problems, as lasers once were. Then there is a fine chapter on the application of self-assembled two-dimensional protein lattices to generate nanocrystalline inorganic lattices by a process here imaginatively entitled "parallel nanometer molecular lithography".

The chemically focused chapters are not easy reading for a materials scientist. They are full of jargon, and no attempt is made to temper the wind to the shorn lamb. This is particularly true of the introductory chapter by the editor (clearly a man with great influence in the whole field). I am driven to the conclusion that the book is intended by its editor for chemically sophisticated readers only, and this is reinforced by the fact that the materials science chapters are concentrated at the rear of the book. By contrast, Calvert's chapter on "Biomimetic inorganic-organic composites" has virtually no jargon of any kind, focuses on actual and possible uses, and reviews the whole field of ceramics and composites in a broad and balanced manner. I am well aware that in making these critical comments I may be doing nothing more than revealing my own lack of chemical expertise, but it remains true that the chapters with an approach like Calvert's are better written.

Despite the severe incidence of highly technical chemistry, a good deal of this book will be accessible and valuable to materials scientists (including ceramists), as well as to chemists intent on novel approaches to synthesis. The book is a pioneering effort, especially in its combined attention to both biosynthesis and biomimetics, and as such is to be welcomed. □

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RAGE and amyloid- β peptide neurotoxicity in Alzheimer's disease

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Amyloid-beta peptide is central to the pathology of Alzheimer's disease, because it is neurotoxic—directly by inducing oxidant stress, and indirectly by activating microglia. A specific cell-surface acceptor site that could focus its effects on target cells has been postulated but not identified. Here we present evidence that the 'receptor for advanced glycation end products' (RAGE) is such a receptor, and that it mediates effects of the peptide on neurons and microglia. Increased expression of RAGE in Alzheimer's disease brain indicates that it is relevant to the pathogenesis of neuronal dysfunction and death.

In Alzheimer's disease, intracellular and extracellular deposits of filamentous proteins are correlated with eventual neuronal dysfunction leading to dementia^{1–4}. Amyloid- β peptide (A β) is the principal component of the extracellular deposits in Alzheimer's disease, in senile or diffuse plaques and in cerebral vasculature. A β promotes neurite outgrowth, generates reactive oxygen intermediates (ROIs), induces cytotoxic cellular oxidant stress, and promotes microglial activation^{1–13}. A β presumably induces these cellular effects by engaging a binding protein (or proteins) presented by plasma membranes. Of the several cell-associated proteins or sulphated proteoglycans that can interact with A β (substance P receptor, the serpin-enzyme complex (SEC) receptor, apolipoproteins E and J, transthyretin, alpha-1 antichymotrypsin, β -amyloid precursor protein, and sulphonates and heparan sulphates), only the substance P and SEC receptors might function as neuronal cell-surface receptors for A β , although there is no direct evidence for this^{6,14–21}. We postulated that at least one other cellular binding site was present that could tether A β to the cell surface. A β , bound at the cell surface, could trigger reactions generating cytotoxic oxidizing stimuli. If this hypothesis is correct, blocking access of A β to the cell-surface binding site(s) might considerably retard progression of cellular dysfunction in Alzheimer's disease.

Here we report the isolation and characterization of a polypeptide of molecular mass ~50K, which binds synthetic A β (1–40 and 1–42). It is identical to RAGE, previously identified as a central cellular receptor for advanced glycation endproducts or AGEs^{22,23}, and also as a neuronal receptor for amphotericin²⁴, a molecule involved in neurite outgrowth²⁵. We show that RAGE mediates the interaction of A β with endothelial cells and neurons, resulting in cellular oxidant stress, and with microglia, resulting in cellular activation (cytokine production, chemotaxis, and haptotaxis). Enhanced expression of RAGE in Alzheimer's disease, in affected neurons, in microglia and in the vasculature, is consistent with the concept that A β –RAGE interaction may contribute to

neurotoxicity that results in dementia.

Oxidant stress markers and A β

Association of neuronal injury in Alzheimer's disease and oxidant stress is manifested by expression of a haem oxygenase type 1 (HO-1), malondialdehyde lysine epitopes, and nuclear localization of the p50 subunit of NF- κ B^{9,26,27}. Neurons adjacent to senile plaques displayed HO-1, and elevated levels of the p50 subunit of the NF- κ B family; p50 was especially prominent in nuclei, suggesting its activation (data not shown). These markers were also in dystrophic neurites contiguous to A β amyloid deposits, though there was little expression in age-matched controls or in areas of Alzheimer's disease brain remote from plaques. Oxidant stress (increased HO-1 and p50) was also evident in those cerebral vessels with accumulated deposits of A β , in both endothelial and smooth muscle cells, but vasculature of age-matched controls stained minimally for HO-1 and p50 (data not shown). We have previously described apparent concentration of HO-1 and p50 in neurofibrillary tangles, and we found induction of binding activity for NF- κ B motifs in nuclear extracts from Alzheimer's disease brain by electrophoretic mobility gel-shift assay²⁷. Here, we note a spatial association between A β plaques and neurons with signs of enhanced cellular oxidant stress in Alzheimer's disease, suggesting that specific binding of A β to cellular surfaces could promote this perturbation.

We postulated that the interaction of A β with both vascular cells and neurons causes oxidant stress and brings about perturbation of cellular function. Incubation of synthetic A β (1–40) with endothelial cells from murine cerebral vasculature, or with PC12 cells, resulted in dose-dependent generation of thiobarbituric acid-reactive substances (TBARS), which was blocked by pre-treatment of cultures with the antioxidants probucol or N-acetylcysteine (Fig. 1a, b). These results are consistent with what has been reported previously about neurons exposed to A β ^{10,28,29}, or with A β -induced perturbation of endothelial cells.

This A β -induced cellular oxidant stress involves interaction with a cell-surface polypeptide, as when endothelial cells or cortical neurons were briefly treated with trypsin, there was virtually complete inhibition of generation of TBARS in the presence of A β (Fig. 1a, b).

Cultured endothelial cells bound 125 I-labelled synthetic A β (1–40) specifically and in a dose-dependent manner, blocked by trypsin (Fig. 1c). Half-maximal binding occurred at 40.0 ± 9.79 nM; at saturation, there were $\sim 1.7 \times 10^4$ molecules bound per cell. Concentrations of 125 I-labelled A β which increased occupancy of cell-surface binding sites were comparable to those inducing endothelial generation of TBARS (Fig. 1a). Similarly, cultured rat cortical neurons displayed 125 I-labelled A β binding that was trypsin sensitive and dose dependent, K_d 55.2 ± 14.6 nM (Fig. 1d).

Crosslinking of surface-bound 125 I-labelled A β on endothelial cells using disuccinimidyl suberate produced a single major band, $M_r \sim 50$ –55K (Fig. 1e, lane 3), whose intensity was greatly attenuated upon addition of an 100-fold molar excess of unlabelled A β (Fig. 1e, lane 2). This band was not due to self-aggregation of the tracer, because when 125 I-labelled A β was incubated with disuccinimidyl suberate (DSS) under the same conditions in the absence of endothelial cells, no band was observed (data not shown). Similar results were obtained with cortical neurons (Fig. 1f).

We then had to determine whether cell-associated 125 I-labelled A β was interacting with previously recognized A β -binding proteins, such as apolipoproteins E and J, heparin-like and heparan sulphate proteoglycans, substance P receptors, and transthyretin^{6,14–21}. Pre-treatment of endothelial cells with heparin ($10 \mu\text{g ml}^{-1}$), which releases cell-associated apoE, or preincubation with heparanase, did not affect 125 I-labelled A β binding. In pilot studies, substance P (added as unlabelled competitor), or antibody to transthyretin, present during the incubation of 125 I-

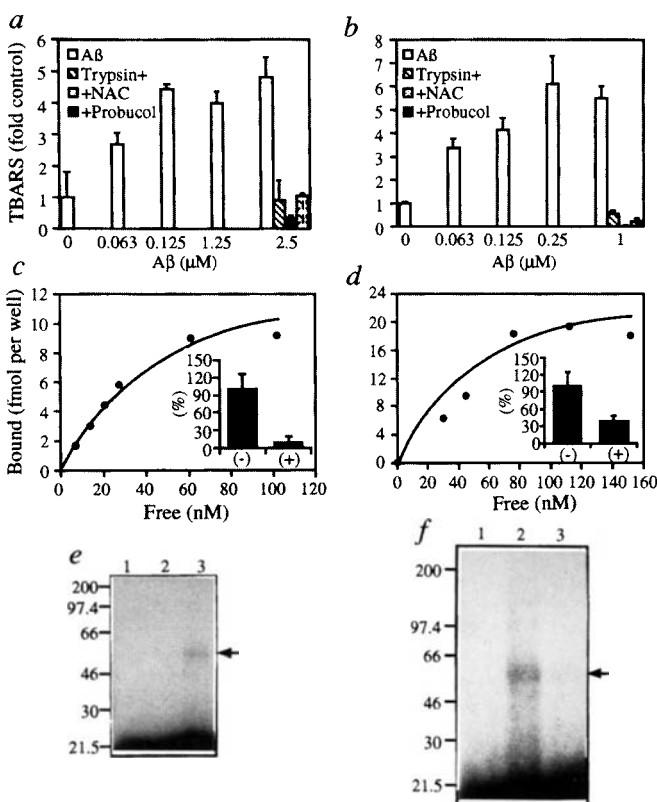
labelled A β with cells, had no effect on binding (data not shown). Apo J, with $M_r \sim 80$ K, was unlikely to be responsible for the ~ 55 K band observed on sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) after crosslinking of 125 I-labelled A β to the cell surface.

To purify cellular A β binding proteins, we developed an assay to measure binding of 125 I-labelled A β extracts of cultured cells or tissues immobilized on microtitre wells. Cultured endothelial cells (whose binding of 125 I-labelled A β was similar to that of neurons) were extracted with octyl- β -glucoside in the presence of protease inhibitors, and the diluted mixture was adsorbed to microtitre wells. Specific binding of 125 I-labelled A β was similar to that observed on cultured endothelial cells: it was dose-dependent (K_d 32.9 ± 10.2 nM) and completely prevented by pre-treatment of the cell extract with trypsin (data not shown). As starting material for purification of cell-associated A β binding proteins, acetone extract of endothelial cell-rich bovine lung was tested using a similar assay. Again, comparable specific, trypsin-sensitive, and dose-dependent binding was observed. Although extract of brain, especially with Alzheimer's disease, would seem more appropriate for purification of A β binding polypeptides, it was not a convenient starting material as binding assays with brain extract were not reproducible, probably because of the high content of lipid, and because human brain was not available in sufficient quantity.

Acetone extract of lung was sequentially chromatographed on heparin and hydroxylapatite columns. Fractions with highest binding activity for 125 I-labelled A β were concentrated and applied to preparative nonreduced SDS-PAGE; two main bands were observed corresponding to M_r of 30–35 and 50–60K (Fig. 2a). Another lane of the same gel loaded with the same sample was sliced, each piece was eluted, and the eluate assayed for 125 I-labelled A β binding; each of the two main protein bands specifically bound 125 I-labelled A β (Fig. 2a). In contrast, eluates of lanes

FIG. 1 A β -induced cellular oxidant stress and binding involves association with a cell-surface, trypsin-sensitive polypeptide on endothelium (a, c, e) and PC12 or cortical neurons (b, d, f). a, c, e, Endothelial cells were incubated with the indicated concentration of synthetic A β (1–40) or 125 I-labelled A β , and cellular oxidant stress was assessed by the TBARS assay (a) or radioligand binding studies were done (c, e). Where indicated, cultures were pre-treated with probucol or *N*-acetylcysteine (NAC), or briefly exposed to trypsin (c, inset: +, trypsin; –, controls). e, Crosslinking of 125 I-labelled A β to the cell surface was done with DSS. Lane 1, 125 I-labelled A β alone + endothelium (no DSS); 2, 125 I-labelled A β with excess unlabelled A β (100-fold), DSS and endothelium; and 3, 125 I-labelled A β with DSS and endothelium. b, d, f, Same experiments with primary cultures of PC12 cells (b) or rat cortical neurons (d, f). f, Samples are: 1 as in e; 2, 125 I-labelled A β with DSS and cortical neurons; and 3, 125 I-labelled A β with excess unlabelled A β , DSS and cortical neurons. Experiments were repeated at least four times, and representative results are shown.

METHODS. Cultured mouse brain microvascular endothelial cells⁴², primary cultures of rat cortical neurons⁴³, or PC12 cells (ATCC) were incubated with A β (1–40; Quality Controlled Biochemicals) and generations of TBARS⁴⁴ measured. For binding studies, A β was radioiodinated (Iodobead, Pierce, Rockville, ILL.), the reaction mixture was desalted by gel filtration, and 125 I-labelled A β migrated as a single band with $M_r \sim 4$ K on Tris or tricine gel (16%) electrophoresis. Binding studies were done immediately after labelling. Cultures were washed with phosphate-buffered saline (calcium and magnesium-free), followed by addition of binding buffer (minimal essential medium containing bovine serum albumin-fatty acid-free (1%)). Tracer, 125 I-labelled A β , was added either alone, in the presence of an 100-fold molar excess of unlabelled A β or other agents as indicated, and cultures were incubated (4°C for 3 and 3.5 h for neurons and endothelial cells, respectively) to allow binding. Unbound ligand was removed by four rapid washes (6 seconds per well). Elution buffer (NaCl, 0.1 M, containing Nonidet P-40, 1%) was then added to wells for 5 min at 37°C , and the liquid aspirated and counted. Specific binding was defined as total binding (125 I-labelled A β in the presence of an 100-fold excess of unlabelled A β), and analysed using nonlinear least-squares analysis. Anti-bovine RAGE IgG interacts with rat, human and bovine RAGE^{24,38}. Some cells were pretreated with TPCK-treated trypsin (0.05 units ml^{-1} for 30 min) and serum or soyabean trypsin inhibitor ($100 \mu\text{g ml}^{-1}$) for added to neutralize trypsin before the binding assay. On endothelial cells and neurons, surface bound



125 I-labelled A β was crosslinked by adding DSS (0.2 mM; Pierce) for 30 min at 25°C . Cultures were then washed twice and dissolved in lysis buffer (NaCl, 0.1 M; NP-40, 1%; PMSF, 2 mM; and leupeptin, $10 \mu\text{g ml}^{-1}$) at 4°C for 1 h. Debris was removed by centrifugation ($10,000 \text{ r.p.m.}$ for 10 min) and the supernatant was prepared for nonreduced SDS-PAGE (10%).

in which molecular mass standards or other fractions from either of the two columns above were run in place of test samples did not bind ^{125}I -labelled A β .

Using the same gel system as above, bands were cut out and subjected to amino-terminal sequence analysis. The sequence of the ~50K band was virtually identical (over 30 cycles) to that of bovine RAGE^{22,23}. On the basis of protein structure deduced from the bovine RAGE complementary DNA and our previous results, the ~50K band probably corresponds to full-length RAGE. The 30–35K band also had the same N-terminal sequence as RAGE, and presumably represented the extracellular domain of RAGE which undergoes proteolysis during purification^{22,23}. Consistent with these data, both 30–35K and ~50KDa polypeptides were reactive with anti-RAGE IgG prepared against recombinant human RAGE (data not shown). A minor, more slowly migrating peak of A β binding activity, corresponding to M_r ~ 66K (Fig. 2a), gave two N-terminal sequences, those for RAGE and albumin. Despite the technical difficulties, a similar series of steps to identify ^{125}I -labelled A β binding activity in brain homogenates also showed RAGE to be an important A β -binding polypeptide (data not shown).

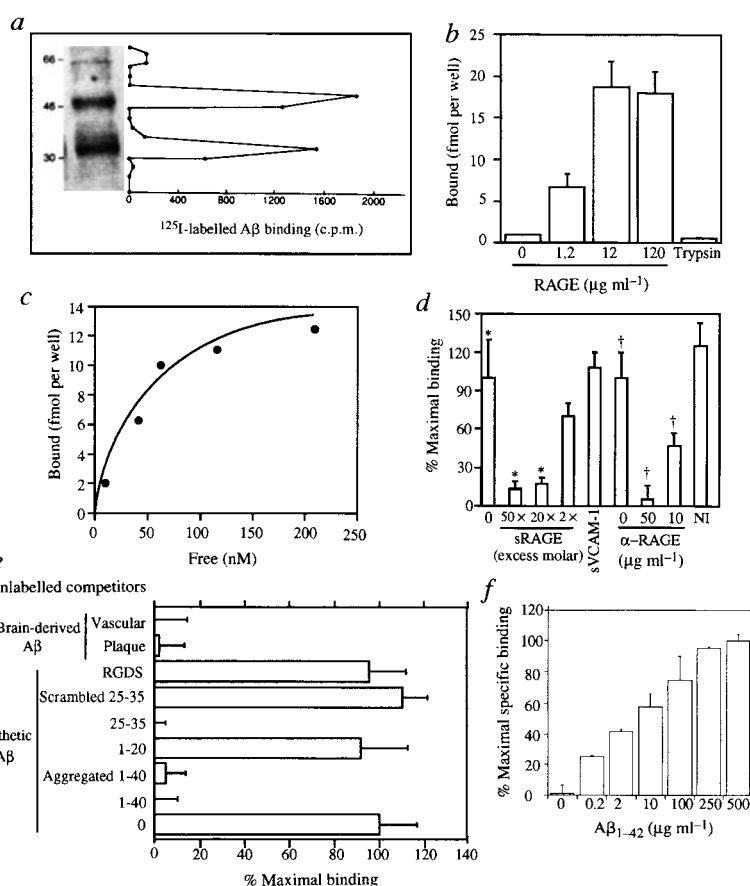
Using the extracellular domain (termed soluble or sRAGE) of purified recombinant human RAGE²⁴ adsorbed to microtitre wells, specific binding of ^{125}I -labelled A β (binding of ^{125}I -labelled A β alone minus that with ^{125}I -labelled A β and excess unlabelled

A β) was dependent on the concentration of RAGE (Fig. 2b). No specific binding was observed when RAGE was replaced by albumin. Exposure of RAGE-coated wells to trypsin completely blocked subsequent binding of ^{125}I -labelled A β , whereas serum or soyabean trypsin inhibitor added to neutralize trypsin had no effect on binding of ^{125}I -labelled A β . When the concentration of RAGE adsorbed to well was held constant, binding of ^{125}I -labelled A β was dose-dependent, being half-maximal at 56.8 ± 13.9 nM (Fig. 2c). This was similar to the range of A β concentrations resulting in binding to and oxidant stress in endothelial cells, PC12 cells and cortical neurons. Addition of either anti-RAGE IgG or soluble RAGE simultaneously with ^{125}I -labelled A β resulted in dose-dependent inhibition of binding (Fig. 2d), whereas non-immune rabbit IgG or another immunoglobulin superfamily molecule, vascular cell adhesion molecule-1 (VCAM-1)³¹, was without effect.

The part of A β (1–40) interacting with RAGE, and the effect of A β purified from Alzheimer's disease brain were then assessed (Fig. 2e). In the presence of freshly-prepared unlabelled A β (1–40), binding of ^{125}I -labelled A β to RAGE was blocked. Competition was also observed if A β (1–40) was preincubated under conditions promoting aggregation of the peptide. A truncated form of A β (1–40) consisting of residues 25–35 also inhibited binding of ^{125}I -labelled A β to RAGE. In accord with the specificity of A β -RAGE interaction, addition of excess unlabelled A β (1–

FIG. 2 Purification of cellular A β -binding polypeptide(s) (a) and characterization of the binding of A β to RAGE (b–f). a, Tissue extract chromatographed on heparin and hydroxylapatite columns. Fractions with peak ^{125}I -labelled A β binding activity from the hydroxylapatite column were subjected to SDS-PAGE (10%; nonreduced), and stained with Coomassie blue or sliced into pieces, eluted, and assayed for specific ^{125}I -labelled A β binding activity. b, c, Binding of ^{125}I -labelled A β (1–40) to purified RAGE immobilized in microtitre wells. b, The indicated concentration of RAGE was incubated for adsorption to wells (^{125}I -labelled A β , 100 nM); and c, the indicated concentration of ^{125}I -labelled A β was added to wells preincubated with RAGE (5 μg per well). Where indicated, wells with adsorbed RAGE were preincubated with trypsin as described above. d, Binding of ^{125}I -labelled A β (100 nM) to RAGE adsorbed to microtitre wells in the presence of the indicated concentration of sRAGE, sVCAM-1 (20-fold molar excess), anti-RAGE IgG or nonimmune IgG (10 μg ml⁻¹). e, Plates were coated with RAGE (5 μg per well) as above, and were then incubated with ^{125}I -labelled A β (100 nM) in the presence of one of the following unlabelled competitors (100-fold molar excess): Arg-Gly-Asp-Ser (RGDS); scrambled A β (25–35); A β (25–35); A β (1–20); aggregated A β (1–40); A β (1–40, fresh); A β -derived from Alzheimer's disease brain (vasculature or plaque); and 0 denotes no addition. f, Binding of ^{125}I -labelled sRAGE (labelled by the iodogen method; 50 nM) to A β (1–42) immobilized on microtitre wells. The indicated concentration of A β was incubated with plates, and then ^{125}I -labelled sRAGE was added alone or with 100-fold excess of unlabelled RAGE. Data show per cent maximal specific binding (mean $\pm$ s.e.m. of 4 experiments). Maximal specific binding (100%) is that observed with ^{125}I -labelled sRAGE (100 nM or the indicated concentration) alone minus ^{125}I -labelled sRAGE in the presence of excess unlabelled sRAGE. Data were analysed by ANOVA Fisher's PLSD and the *P* values are * < 0.01; † < 0.02. Experiments were repeated a minimum of four times, and representative results are shown.

METHODS. Bovine lung extract dissolved in Tris-buffered saline containing octyl- β -glucoside (1%) and PMSF (2 mM) was applied to heparin hyperD (bed volume 40 ml; Biosepra), the column was eluted with increasing concentrations of NaCl, and fractions with maximal ^{125}I -labelled A β binding activity identified. The latter were loaded on hydroxylapatite ultragel (20 ml bed volume; Biosepra), the column was eluted with increasing concentrations of phosphate, and fractions with maximal ^{125}I -labelled A β binding activity were applied to preparative nonreduced SDS-PAGE (10%). Gels were stained with Coomassie blue, and identical lanes of the gel were cut into 2-mm slices from which protein eluted was studied for A β binding



activity, or slices of the gel were subjected to N-terminal sequence analysis²⁴. Human recombinant sRAGE was obtained from a lung library by PCR, expressed using baculovirus, and purified as described²⁴. Other additions included synthetic peptides, sVCAM-1, antibodies to sRAGE^{24,45}, or chromatographically purified A β from Alzheimer's disease brain³¹. The first three immunoglobulin-like domains of VCAM-1 were obtained from a lung cDNA library using PCR (primers were 5'GAAATGCTGTGAAGATGTCG3' and 5'CTCATGAACTAATCCACTTC3'), expressed in a baculovirus system and purified as above for sRAGE except that Q Sepharose was used.

20), scrambled A β (25–35), or an irrelevant peptide such as Arg-Gly-Asp-Ser had no effect on 125 I-labelled A β binding to RAGE (Fig. 2e). Furthermore, human A β (1–40) or (1–42), chromatographically purified form plaques or vascular amyloid from Alzheimer's disease patients³², inhibited the binding of 125 I-labelled A β (Fig. 2e). Although these data show that A β binds to RAGE, they do not allow us to discriminate whether monomer, low or higher molecular mass oligomers are the preferred or the relevant ligands. To test whether RAGE would bind directly to insoluble A β (1–42), we adsorbed the peptide to microtitre wells, and then assayed binding with 125 I-labelled sRAGE (Fig. 2f). Incubation of wells with increasing concentrations of A β (1–42), resulted in increased binding of 125 I-labelled sRAGE; binding was blocked by inclusion of excess unlabelled soluble RAGE. To be certain

that binding of 125 I-labelled A β to RAGE reflected a specific interaction, studies were done with two other known ligands of RAGE, AGE albumin and amphoterin. Binding of 125 I-labelled A β (1–40) to RAGE was blocked in a dose-dependent manner by addition of increasing amounts of AGE albumin or amphoterin (data not shown), consistent with the postulate that each of these ligands interacts with contiguous or overlapping portions of the receptor. Another possibility was that generation of oxygen free radicals, a property of A β ⁹ and AGE²⁷ ligands of RAGE, might mediate A β –RAGE interaction; however, addition of antioxidants, such as probucol, had no effect. Finally, it was important to determine whether AGE-like determinants, potentially present in Alzheimer's disease-derived A β might contribute to A β –RAGE interaction. Affinity-purified anti-AGE IgG, previously shown to bind AGEs and to prevent their interaction with RAGE³³, did not alter binding of A β to RAGE (data not shown). Thus, structural determinants on A β that are distinct from AGEs, and are yet to be identified, are critical to A β –RAGE interaction.

Confirming that RAGE could mediate the binding of A β to cells, COS-1 cells transfected with a RAGE-containing construct²³ demonstrated specific binding of 125 I-labelled A β ($K_d \pm 6.2$ nM; Fig. 3a). Mock-transfected COS-1 cells bound 125 I-labelled A β minimally. In the more relevant cultured mouse brain endothelial cells (Fig. 3b, c) and rat cortical neurons (data not shown), binding of 125 I-labelled A β was blocked by addition of excess soluble RAGE or anti-RAGE IgG in a dose-dependent manner. Excess soluble VCAM-1 or irrelevant antibodies which bind to the surface of each cell type (anti-mouse thrombomodulin IgG for endothelial cells and anti-mouse NCAM-1 for neurons) did not inhibit 125 I-labelled A β binding. These data indicate that RAGE is a binding site for 125 I-labelled A β on cellular surfaces.

RAGE and A β -induced cellular stress

A β binding to RAGE and A β -induced cellular perturbation resulted in oxidant stress and cytotoxicity. RAGE-transfected COS-1 cells generated more TBARS than mock-transfected controls (Fig. 3d). RAGE can mediate A β -induced oxidant stress on endothelium and neuronal cells. Endothelial cells incubated with A β generated TBARS (Figs 2a, 3e), which was prevented by blocking access to RAGE using either anti-RAGE IgG or excess soluble receptor (Fig. 3e; antibody to thrombomodulin or nonimmune IgG was without effect). Activation of NF- κ B was also suppressed by blocking RAGE (Fig. 3f, compare lanes 3 and 4). Anti-thrombomodulin IgG did not affect this A β -mediated oxidant stress (data not shown). PC12 cells exposed to A β also manifested generation of TBARS and activation of NF- κ B, prevented in large part by blocking interaction of A β with the receptor.

RAGE-transfected COS-1 cells incubated with A β (25–35) had diminished ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide¹⁰ (MTT), whereas mock-transfected controls required much higher concentrations of A β for similar suppression of MTT reduction (Fig. 4a). Thus, expression of RAGE increases vulnerability to A β ; but, suppression of MTT reduction in mock-transfected COS cells at higher concentrations of A β (> 1 μ M) indicates that other mechanisms are also likely to be involved. PC12 cells exposed to A β (25–35) exhibited dose-dependent attenuation of MTT reduction which was largely prevented by blockade of RAGE with either sRAGE (Fig. 4b, c) or anti-RAGE IgG (data not shown). In parallel, morphological changes caused by A β , including retraction of cellular processes, were also prevented (Fig. 4d–f; compare e with f, and with control cultures in d).

The above data indicated that RAGE, if present and/or up-regulated in cells important in the pathogenesis of Alzheimer's disease, could mediate toxic effects when associated with A β . Enzyme-linked immunosorbent assay (ELISA) of homogenates of Alzheimer's disease brain for RAGE demonstrated roughly a 2.5-fold increase in patients compared with age-matched controls (Fig. 5e). Immunocytochemical studies showed apparently

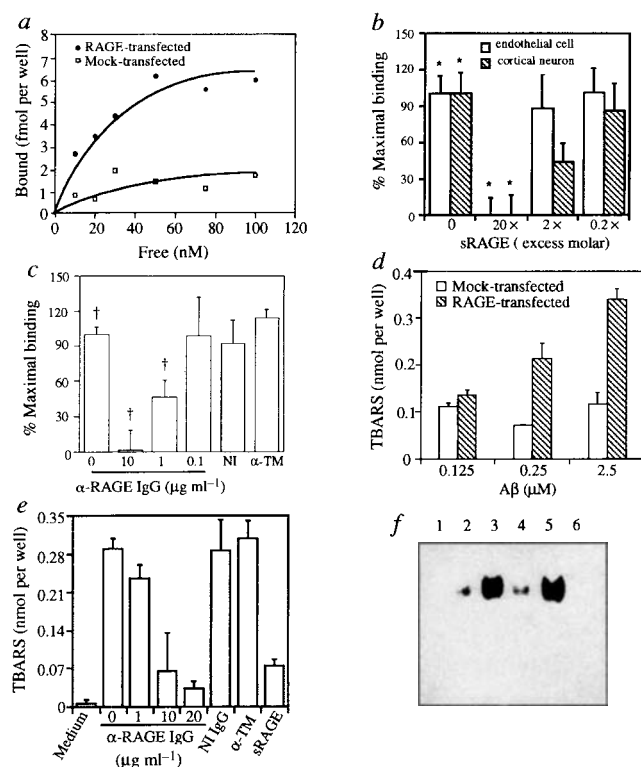
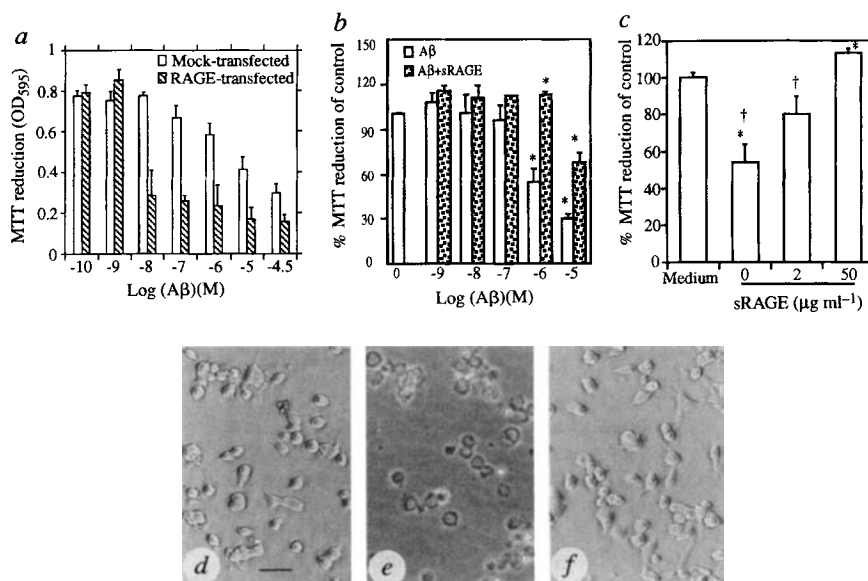


FIG. 3 a, Binding of 125 I-labelled A β to RAGE-transfected COS-1 cells. b, Effect of sRAGE on binding of 125 I-labelled A β to endothelium and cortical neurons. c, Effect of anti-RAGE IgG, at the indicated concentration, on binding of 125 I-labelled A β to endothelium; cell cultures were incubated with 125 I-labelled A β (100 nM) alone or in the presence of the indicated concentrations of sRAGE, and anti(α -RAGE, anti-thrombomodulin (20 μ g ml⁻¹) or nonimmune IgG (NI; 20 μ g ml⁻¹), (mean $\pm$ s.e.m. of 4 experiments). P values shown are † <0.01 and * <0.05. d, e, Generation of TBARS in RAGE-transfected COS-1 cells (d) and endothelial cells (e) incubated with A β (1 μ M or indicated concentration) either alone or in the presence of anti-RAGE IgG, and anti-thrombomodulin IgG (α -TM; 20 μ g ml⁻¹), nonimmune IgG (NI; 20 μ g ml⁻¹), or sRAGE (100 μ g ml⁻¹). P values shown are * <0.01 and † <0.05. f, Activation of NF- κ B in endothelial cells incubated with A β alone or with other additions (as above). Nuclear extracts were then prepared and studied by EMSA: Lane 1, 32 P-labelled NF- κ B probe; 2, cells not exposed to A β ; 3, cells exposed to A β (500 nM); 4, 5, cells exposed to A β (500 nM); 4) in the presence of anti-RAGE IgG or nonimmune IgG (10 μ g ml⁻¹ in each case; lane 5); 6, extracts of cells exposed to A β (500 nM) in the presence of 100-fold excess unlabelled NF- κ B probe. Experiments were repeated a minimum of four times, and representative results are shown. METHODS. Binding of 125 I-labelled A β and A β -mediated generation of TBARS and activation of NF- κ B were studied as described in Fig. 1. Nuclear translocation of NF- κ B was studied by EMSA with a consensus 32 P-labelled human NF- κ B probe²⁷. Transfection of COS-1 cells with the RAGE cDNA was as described²².

FIG. 4 Effect of RAGE blockade on A β -induced cellular stress in RAGE-transfected COS-1 cells (a) and PC12 cells (b–f). Cultures were exposed to the indicated concentrations of A β (25–35) alone, or in the presence of either sRAGE, anti(α) RAGE IgG, or nonimmune (NI) IgG. a, MTT reduction by RAGE-transfected or mock-transfected COS-1 cells (procedure described by Promega) is shown at the indicated concentrations of A β after overnight incubation. b, c, The effect of RAGE blockade on MTT reduction in PC12 cells is shown. b, MTT reduction at different concentrations of A β in the presence or absence of sRAGE (20-fold molar excess over A β) and c, MTT reduction at different concentrations of sRAGE (A β , 1 μ M). P values * <0.01 and † <0.05. d–f, Micrographs (PX-10 microscope) depicting PC12 cells incubated in medium alone (d) or medium containing the following: A β (1 μ M; e); or A β (1 μ M) + sRAGE (50 μ g ml⁻¹; f). Scale bar, 100 μ m. Experiments were repeated a minimum of five times, and representative results are shown.



increased expression of RAGE (Fig. 5a) in neurons with signs of oxidant stress (data not shown) especially in those close to deposits of A β . In contrast, RAGE expression was minimal in age-matched controls (Fig. 5b). RAGE expression was also enhanced in vessel walls (Fig. 5c) near A β deposits in Alzheimer's disease brain (Fig. 5c inset shows staining for RAGE alone), whereas vasculature in age-matched control brain showed little RAGE expression (Fig. 5d). Thus, RAGE expression would seem to be significantly upregulated in neurons around or participating in the neuritic plaques, as well as in the vasculature of the affected Alzheimer's disease brain. Further study will be required to localize precisely these changes in RAGE expression in different areas of the brain.

RAGE mediates microglial activation by A β

Microglia, identified by immunostaining for CD68 in Alzheimer's disease brain, close to senile plaques, showed strong expression of

RAGE antigen (Fig. 6a, b). RAGE was also present in cultured rat brain microglia, as well as in the immortalized mouse microglial cell line, BV-2 (ref. 34; data not shown). RAGE on BV-2 cells binds ¹²⁵I-labelled A β . This was dose-dependent, with K_d 25.1 $\pm$ 9.0 nM (Fig. 6c). Blockade of RAGE, with either excess sRAGE or anti-RAGE IgG or F(ab')₂, prevented binding of ¹²⁵I-labelled A β to microglia, but neither nonimmune rabbit IgG or F(ab')₂, nor anti-Mac 1 IgG (which binds to BV-2 cells) affected this interaction (data not shown). Binding of ¹²⁵I-labelled A β (1–40) to BV-2 cells was competed by addition of excess unlabelled A β (1–40 or 25–35), but not by A β (1–20) or by scrambled A β (25–35), or the unrelated peptide RGDS; unlabelled A β derived from vascular or plaque amyloid from Alzheimer's disease brain was also inhibitory. Thus, characteristics of the binding of ¹²⁵I-labelled A β to BV-2 cells were similar to those observed above for A β interaction with purified RAGE, or RAGE on endothelial and PC12 cells.

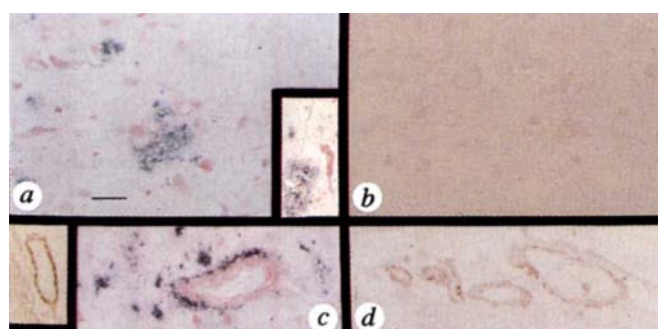


FIG. 5 Localization of RAGE in neurons and vasculature near deposits of A β in Alzheimer's disease brain. a, Increased expression of RAGE in temporal lobe from Alzheimer's disease brain. Inset in a depicts RAGE antigen also in the neuritic processes. b, Staining for RAGE in age-matched control brain. c, RAGE in vasculature from temporal lobe in Alzheimer's disease brain (inset, single staining of RAGE in Alzheimer's disease vasculature). d, Staining for RAGE in vasculature from age-matched temporal lobe control. Except as indicated above, each was double stained: the red colour shows RAGE, and the black colour reveals A β . RAGE was similarly distributed in hippocampus (data not shown). Scale bar, 50 μ m (a–c, inset), and 30 μ m (d). Immunohistological studies are representative of 7 Alzheimer's disease and 5 age-matched controls. e shows ELISA for RAGE antigen in homogenates of Alzheimer's disease brain (n = 20) versus age-matched apparently normal control (n = 10).

METHODS. Immunohistological studies were done on paraffin sections of formalin-fixed temporal lobe from Alzheimer's disease and age-matched controls^{27,45}. Sections were first stained with either rabbit anti-human RAGE IgG (20 μ g ml⁻¹), and sites of binding of primary antibody were visualized using the avidin–biotin alkaline phosphatase method. Double staining of these sections for A β was done using murine monoclonal anti-human A β IgG (5 μ g ml⁻¹; J. Goldman, Columbia), followed by peroxidase-conjugated goat anti-mouse IgG. Nonimmune rabbit or mouse IgG were used as controls.

A β -RAGE interaction induces migration of microglia. Addition of A β (1-40) or A β (25-35) to BV-2 cells in microchemotaxis chambers resulted in directional cell migration, dependent on a concentration gradient of the stimulus from upper to lower chamber⁷ (data not shown). Cell migration was prevented by addition of anti-RAGE F(ab')₂, but not by nonimmune IgG or F(ab')₂ (Fig. 6d) or anti-Mac 1 IgG. Anti-RAGE IgG or F(ab')₂ inhibited A β -induced migration, but it had no effect on motility of BV-2 cells induced by formylated peptide (formyl-methionyl-leucyl phenylalanine, fMLP; Fig. 6d). Microglia accumulate at deposits of A β ^{35,36}, probably because, in contrast to soluble A β , immobilized A β blocks microglial migration (haptotactic response). When either A β or albumin was adsorbed to the upper surface of chemotaxis chamber membranes and BV-2 cells were added, cell migration across A β -coated membranes was diminished in response to a range of fMLP concentrations added to the lower compartment, whereas albumin-coated membranes allowed free passage of cells, comparable to untreated membranes (data not shown). Blockade of RAGE with anti-RAGE F(ab')₂ (Fig. 6e) or with excess sRAGE (data not shown) enhanced BV-2 migration across A β -coated membranes to levels observed in membranes coated with albumin in response to fMLP added to the lower compartment, whereas nonimmune IgG or F(ab')₂, or anti-Mac IgG was without effect. These data indicate that soluble A β induces microglial migration whereas immobi-

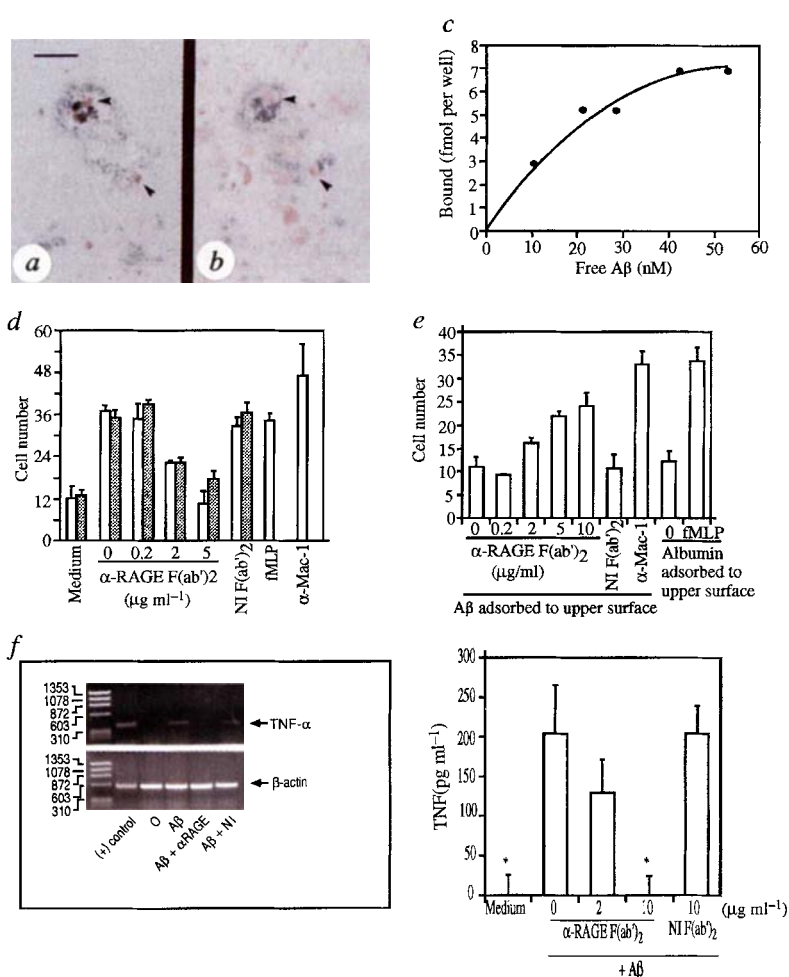
lized A β arrests microglial migration, in each case largely because of engagement of RAGE by A β .

Activated microglia, which can produce cytotoxic and other cytokines^{11,13,36,37}, may be another important source of neuronal damage in Alzheimer's disease. A β can stimulate microglial expression of tumor necrosis factor- α (TNF α)¹¹. This may be especially significant, as TNF α could recruit additional host effector mechanisms. A β incubated with BV-2 cells induced TNF α transcripts as demonstrated by polymerase chain reaction (Fig. 6f), followed by elaboration of TNF α into culture supernatants (Fig. 6f). Blockade of RAGE, with anti-RAGE F(ab')₂, prevented the appearance of TNF α messenger RNA and diminished TNF α antigen to levels seen in untreated cells, whereas nonimmune F(ab')₂ was without effect. Similarly, A β -enhanced activation of NF- κ B in BV-2 cells was largely blocked by addition of anti-RAGE F(ab')₂, but not by nonimmune F(ab')₂, or by the antioxidant probucol (data not shown).

Discussion

RAGE is a member of the immunoglobulin superfamily of cell surface molecules originally identified by its capacity to bind advanced glycation endproducts^{22,23}. Engagement of these non-enzymatically glycated adducts by RAGE on endothelial cells and mononuclear phagocytes mediates cellular perturbations having important consequences for vascular homeostasis^{33,37,38}.

FIG. 6 RAGE and microglial cells. *a, b*, RAGE in microglial cells. Co-localization of CD68 (*a*; mouse anti-human CD68 IgG, 4 μ g ml⁻¹), RAGE (*b*; rabbit anti-human RAGE IgG, 30 μ g ml⁻¹), and A β (rabbit anti-A β IgG (1:50 dilution), in *a*, or mouse anti-A β IgG (1:500 dilution), in *b*) in a plaque from Alzheimer's disease brain (adjacent sections). The red colour shows the antigen (CD68 or RAGE) and the black colour depicts localization of A β . Scale bar, 100 μ m. *c*, BV-2 cells bind [¹²⁵I]-labelled A β . *d, e*, Role of RAGE in modulation of BV-2 migration by A β . *d*, Effect of anti-RAGE F(ab')₂ or nonimmune F(ab')₂ on A β (1-40, white bars, and 25-35, shaded bars)-induced chemotaxis. *e*, Anti-RAGE F(ab')₂ blocks the haptotactic response to A β (1-40) adsorbed to the upper surface of chemotaxis chamber membranes. BV-2 cells were preincubated with either anti-RAGE F(ab')₂ or nonimmune (NI) F(ab')₂ (10 μ g ml⁻¹) for 1 h at 4 °C, washed then added to the upper compartment of chemotaxis chambers in which the upper surface of membranes were coated with A β (1-40). fMLP (1 μ M) was added to the lower chamber in each case. *f*, Role of RAGE in A β -induced TNF α production in BV-2 cells. BV-2 cells (10⁶) were incubated with A β (25-35; 1 μ M) for 4 h at 37 °C, and RNA was collected. RT-PCR (left) was done using primers for murine TNF α (Clontech) and the housekeeping gene mouse β -actin (Clontech) was simultaneously amplified. The positive control (the respective cDNA) and migration of standards are shown. Where indicated, BV-2 cells were pre-incubated with anti-RAGE F(ab')₂ or nonimmune (NI) F(ab')₂ (10 μ g ml⁻¹ in each case) for 1 h at 37 °C. Right, A β (25-35; 1 μ M) induction of TNF α antigen (Biosource) in BV-2 cells was studied after 18 h incubation at 37 °C with either medium, A β alone or A β in the presence of anti-RAGE F(ab')₂ or nonimmune (NI) F(ab')₂. **P* < 0.005. METHODS. Double immunostaining was done using the indicated primary antibodies as described^{27,45}. Radioligand binding studies used BV-2 cells (5 $\times$ 10⁴ per well) in minimal essential medium with bovine serum albumin (1%) incubated with either [¹²⁵I]-labelled A β alone (1.3 $\times$ 10⁴ c.p.m. ng⁻¹) or in the presence of one of the indicated additions at 4 °C for 3 h. Wells were washed seven times, and cell-associated radioactivity was eluted with Nonidet P-40 (0.5%). Specific binding is shown and equilibrium binding data were analysed as above (Fig. 1). For chemotaxis assays, BV-2 cells (5 $\times$ 10³ per well) were added to the upper compartment of microchemotaxis chambers for 4 h at 37 °C, and then cells reaching the lower surface of the chemotaxis chamber membrane were quantitated per high-power field as described³⁶. Where indicated, the upper surface of the membranes was coated for 3 h at 37 °C with A β (1-40)



or A β -sRAGE by floating either in a solution of A β alone (50 nM) or A β (50 nM) preincubated for 2 h at 37 °C with sRAGE (1 μ M)³⁶. For certain studies, BV-2 cells were pre-incubated with anti-RAGE F(ab')₂ or nonimmune F(ab')₂ (10 μ g ml⁻¹) for 1 h at 37 °C and then washed twice before exposure to A β . These experiments were repeated a minimum of three times.

Expression of RAGE is also especially prominent in the central nervous system. During the embryonic and early postnatal period in rat brain, RAGE is highly expressed by cortical neurons in hippocampus (postnatal day P5) and cerebellum (P17) (ref. 24). AGEs are almost certainly not the primary ligands of RAGE, and in the central nervous system the ligand which can engage RAGE and affect cellular function is amphoterin, a high mobility group I DNA-binding protein strongly expressed in developing neurons and shown to mediate neurite outgrowth when present extracellularly²⁵. Amphoterin binds to RAGE specifically and with relatively high affinity ($K_d \sim 9$ nM), and RAGE is the receptor that mediates its binding to cortical neurons, and subsequent induction of neurite outgrowth²⁴. The receptor has more limited expression in the adult central nervous system⁷ compared with the perinatal period. In adult animals, RAGE is found in a population of cortical neurons yet to be characterized, and in spinal motor neurons. In Alzheimer's disease, expression of RAGE within cortical neurons increases and is more widespread, especially in those neurons close to deposits of A β and those cells bearing neurofibrillary tangles. This suggested that there was yet another ligand for RAGE in Alzheimer's disease brain.

A β itself has been shown to generate ROIs⁹, and A β interaction with PC12 cells additionally triggers intracellular formation of oxidants¹⁰, such as hydrogen peroxide, resulting in perturbation of a range of cellular properties. We reasoned that engagement of A β by a cell-surface polypeptide would tether A β to the membrane where it could exert its oxidant effects in closest proximity to cellular structures, thus evading antioxidant mechanisms prevalent in the extracellular space. Consistent with this concept, binding of A β to cortical neurons paralleled the induction of cellular oxidant stress, and both were blocked by pre-treatment with trypsin. Investigations to understand the role of RAGE in brain, and those to identify cellular binding site(s) for A β that mediate induction of cellular oxidant stress, converged when the cellular A β -binding protein proved to be identical to RAGE. Experiments with recombinant RAGE and blocking antibody confirmed that RAGE specifically bound A β and mediated, in large part, A β -induced oxidant stress.

A sufficient molar excess of the extracellular domain of RAGE (sRAGE) completely blocks ¹²⁵I-labelled A β binding to endothelial cells and cortical neurons, and also completely prevents A β -induced neuronal toxicity. At higher levels of A β (for example, 10 μ M), however, sufficient concentrations of soluble receptor could not be achieved for complete neuroprotection. Anti-RAGE IgG prevented ¹²⁵I-labelled A β binding to cortical neurons by only about 70%, whereas under the same conditions it completely blocked ¹²⁵I-labelled A β binding to endothelial cells. These results

with anti-RAGE IgG indicate that other binding sites may also be involved in A β -neuronal interaction, in addition to RAGE. It is important to note that sRAGE, once bound to A β , seems to block cellular interactions of amyloid- β peptide, whether the latter is present as monomer or predominately as aggregates. We suggest that sRAGE can diminish cellular interactions and toxicity of A β deposits by pre-empting neuronal interactions with cell-surface RAGE and, potentially, other cell-surface binding sites.

A β is an oxidizing agent⁹ (as are AGEs)^{27,39}, and such ligands could exert their cellular effects simply as the result of being tethered to RAGE on the cell surface. However, preliminary studies have shown that stimulation of this receptor by other ligands which do not themselves generate ROIs (A.M.S. and D.S., unpublished observation), induces intracellular generation of oxidants in target cells, suggesting that signal transduction mechanisms may be recruited after ligand-RAGE interaction. Further support for a relationship between RAGE and ROIs is the presence of two functional NF- κ B sites in the RAGE promoter whose activity can be triggered by oxidants (A.M.S., unpublished observation). These data suggest a possible feedback loop whereby binding of A β to RAGE induces oxidant stress which further upregulates RAGE, thereby augmenting further A β -RAGE interaction.

RAGE is an important receptor for A β also on microglial (BV-2) cells. Binding of soluble A β to RAGE induces microglial migration along a concentration gradient, possibly leading to a deposit of insoluble A β . But, immobilized A β (A β fibrils) arrests cell migration, permitting microglia to undergo the sustained activation which may underlie a chronic inflammatory component in Alzheimer's disease^{40,41}. We have found that A β adsorbed to a substrate does indeed induce microglial activation, and the time course for TNF production is more prolonged than that observed after exposure of microglia to soluble A β .

Although these studies represent only a first step in establishing a link between expression of RAGE and pathophysiological changes in Alzheimer's disease, they suggest a novel mechanism in which engagement of RAGE has quite different outcomes depending on the nature on the ligand, cell maturation and the microenvironment. During the fetal and perinatal periods, amphoterin-RAGE interaction promotes neurite outgrowth and supports normal development. Subsequently, RAGE might be a physiological receptor for low levels of A β produced throughout life, perhaps effecting their disposal. However, engagement of RAGE on cortical neurons in the presence of higher levels of A β , especially in aggregated deposits in Alzheimer's disease, could result in sustained cellular oxidant stress with deleterious consequences for cellular function and organic homeostasis. $\square$

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A lower limit of 9.5 Gyr on the age of the Galactic disk from the oldest white dwarf stars

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WHITE dwarf stars represent the final evolutionary state for most main-sequence stars. They cool slowly enough that even the oldest white dwarfs are still observable in sufficiently deep surveys and they therefore provide a record of the age and star-formation history of the local disk of the Milky Way¹⁻⁷—and hence a useful constraint on the age of the Galaxy itself. Here we report the initial results of a very deep survey of white dwarfs, that avoids many of the problems associated with the incompleteness of earlier surveys. We use model age–luminosity relations to interpret the luminosity function of our sample of stars, and thus obtain a minimum age for the local Galactic disk of ~ 9.5 Gyr. Our results lend weight to an emerging picture of the evolutionary history of the Milky Way, in which the halo formed ~ 14 – 17 Gyr ago^{8,9}, followed by the bulge globular clusters ~ 12 – 14 Gyr ago¹⁰, with a modest hiatus before the onset of star formation in the local disk ~ 10 Gyr ago.

Intrinsically faint stars like white dwarfs (WDs) are much easier to find when they share proper motion with companions that are brighter or of contrasting colour. For example, our classification spectroscopy of a sample of ~ 500 wide binaries¹¹⁻¹³ has identified many of the coolest known WDs with apparent magnitudes $m_{pg} \geq +17$ and ~ 250 of the WDs listed in the newest edition of the Villanova catalogue¹⁴. For the present study of the white-dwarf luminosity function (WDLF) we have used charge-coupled-device image data obtained on 13–16 December 1991 (Lowell Observatory), 23–25 August 1992 (Cerro Toldo Inter-American

Observatory, CTIO), 13–18 February 1993 (CTIO) and 9–12 April 1993 (McDonald Observatory). Filters approximating the standard Johnson B, V and Kron–Cousins R, I bandpasses were used at all sites. Standard stars were observed throughout each night to provide extinction and transformation corrections.

Firm spectroscopic identifications and good quality $V-I$ colour indices are available for the 50 WD components listed in Table 1. For each component we computed the bolometric luminosity $\log(L/L_{\odot})$ by assuming a mass of $0.6M_{\odot}$, and interpolating in tables of recently published hydrogen-rich or helium-rich model atmospheres¹⁵, as appropriate for each WD's spectral type.

Determination of the WDLF requires a sample which is known to be complete within well defined limits, or one whose incompleteness is a well behaved function of observable quantities such as apparent magnitude, m_{pg} , and proper motion, μ . We have assessed the completeness of our sample in earlier work^{12,16,17}, within the limits $\mu \geq 0.16$ arcsec yr⁻¹ and $m_{pg} \leq +18.5$, it appears to be $\sim 10\%$ complete. However our strategy for correcting for incompleteness gives us an effective search volume which is at least 50 times larger than is typical of other WD surveys. Thus truly rare WDs, of low luminosity and/or small velocity relative to the Sun, are more likely to be included in our sample.

Table 1 lists each star's raw (indicated by subscript 'r') and corrected (subscript 'c') contribution to the WDLF. The resulting corrected WDLF in the theoretical plane ($\log \phi$ versus $\log(L/L_{\odot})$) is shown in Fig. 1 with filled circles. This WDLF, corrected for incompleteness, suggests that the space density of WDs found in binaries is $\sim 5.3^{+3.5}_{-0.7}$ per 10^3 pc³. After allowing for the fraction that are components of binaries (10 out of 43), the space density of single WDs implied by the sample of Liebert, Dahn and Monet (ref. 7) is $\sim 2.3^{+1.2}_{-0.1}$ per 10^3 pc³. We conclude that the total space density of all WDs in the solar neighbourhood is $\sim 7.6^{+3.7}_{-0.7}$ per 10^3 pc³. Assuming a mean WD mass of $0.6M_{\odot}$, our WDLF implies a mass density of $\sim 4.6^{+2.2}_{-0.4} M_{\odot}$ per 10^3 pc³—more than twice the previous estimate⁷ for the WD contribution to the dynamical mass of the Galaxy. Also, our lowest luminosity bin suggests that faint WDs are five to six times more numerous than estimated in ref. 7—in agreement with an independent search for cool WDs being conducted in the Southern Hemisphere¹⁸.

In order to interpret our observed WDLF, we used the age–luminosity relations from WD evolutionary models computed specifically for this work. These models include more accurate

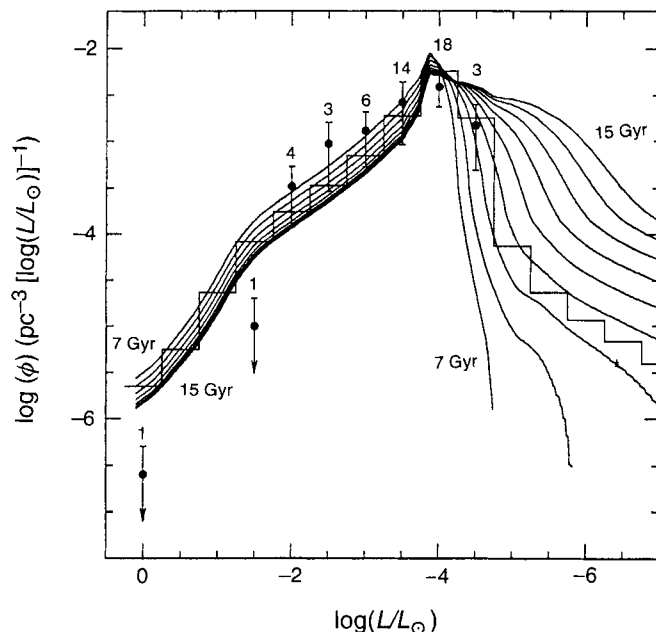


FIG. 1 Comparison of our transformed WDLF (filled circles) and our computed theoretical WDLFs for Galactic disk ages from 7 to 15 Gyr. The observed number of objects is listed at the top of each plotted bin. Completeness correction factors have been computed for each star according to its proper motion μ and apparent magnitude m_{pg} . The inverse of each star's completeness factor is then used as its weight in the space density sums. In effect this counts the stars in each (m_{pg}, μ) bin that are missing due to the incompleteness of the sample¹⁶. In computing space densities, we have also taken into account the fraction of the sky covered (0.651), the fraction of wide binary components that are spectroscopically identified WDs (0.332) and the fraction for which we now have photometric data (0.203). The best fit to the completed WDLF (binned histogram) implies a minimum time of $\sim 9.5^{+1.1}_{-0.8}$ Gyr since the onset of star formation in the solar neighbourhood. All theoretical WDLFs have been normalized to our observed space density of ~ 5.3 stars per 10^3 pc³.

TABLE 1 Photometry of white dwarfs in wide binaries

Name	V	$\sigma(V)$	$(V-I)$	$\sigma(V-I)$	Comp.	$(1/V_m)_r$	$(1/V_m)_c$	$\log(L/L_\odot)$	Source
LP587 – 53	17.48	0.02	0.70	0.03	H	7.38E – 06	7.54E – 05	–3.715	C
G244 – 36	14.03	0.03	0.30	0.04	H	5.91E – 05	1.22E – 03	–3.120	L
LP356 – 88	17.44	0.03	1.22	0.04	H	9.32E – 05	1.33E – 03	–4.394	L
LP889 – 20	18.50	0.02	0.96	0.03	He	1.02E – 05	1.75E – 04	–3.996	C
Omi 2 Eri B	9.31	0.05	–0.33		H	3.04E – 07	3.04E – 07	–0.170	ref. 36
LP775 – 53	17.39	0.03	0.90	0.10	H	1.45E – 05	9.57E – 05	–3.958	C
LP358 – 525	15.78	0.05	0.82		H	6.37E – 05	1.08E – 03	–3.859	refs 36–38
G102 – 39	14.10	0.01	0.56	0.02	He	2.17E – 04	3.68E – 03	–3.582	T
LP600 – 42	15.64	0.02	0.94	0.05	H	1.73E – 04	5.32E – 03	–4.008	C
G87 – 29	15.61	0.05	0.47	0.06	He	4.51E – 06	2.79E – 05	–3.444	L
G107 – 70	14.65	0.05	1.02	0.06	He	6.13E – 05	6.13E – 05	–4.039	L
L186 – 119	16.59	0.02	0.27	0.03	H	4.57E – 06	1.63E – 04	–3.070	C
G116 – 16	15.38	0.01	0.26	0.02	H	8.09E – 06	1.83E – 04	–3.053	T
LP487 – 25	18.43	0.10	1.06	0.11	He	1.57E – 05	1.06E – 04	–4.067	L
G161 – 36	14.76	0.01	–0.10	0.01	H	2.51E – 06	6.18E – 05	–2.329	C
LP462 – 56A	14.85	0.01	0.18	0.01	He	4.93E – 06	5.62E – 05	–2.884	C
LP462 – 56B	15.21	0.01	0.34	0.01	H	7.10E – 06	8.10E – 05	–2.884	C
LP488 – 19	18.18	0.03	0.94	0.04	He	6.43E – 06	7.47E – 05	–3.981	L
LP370 – 50	17.34	0.03	0.45	0.04	H	3.00E – 06	8.65E – 05	–3.363	L
LP370 – 51	17.49	0.03	0.49	0.04	H	2.96E – 06	8.54E – 05	–3.363	L
LP549 – 33	16.10	0.10	0.58	0.12	H	9.97E – 06	1.25E – 04	–3.559	L
LP549 – 32	17.77	0.10	1.15	0.12	He	2.27E – 05	1.80E – 04	–4.124	L
LP791 – 55	15.25	0.03	1.53	0.04	He	4.22E – 04	4.22E – 04	–4.333	T
LP214 – 38	16.95	0.03	0.59	0.04	He	1.14E – 05	1.28E – 04	–3.625	L
LP849 – 59	17.64	0.02	0.98	0.03	He	1.25E – 05	1.07E – 04	–4.011	C
LP552 – 49	17.59	0.03	1.26	0.04	He	6.17E – 05	1.15E – 03	–4.190	L
G148 – 7	13.74		–0.18		H	5.70E – 06	1.89E – 04	–1.897	refs 36–38
LP320 – 643	16.99	0.03	0.73	0.04	He	1.04E – 05	2.08E – 04	–3.795	L
LP497 – 29	18.67	0.10	0.69	0.12	He	3.23E – 06	4.34E – 05	–3.751	L
G14 – 58	12.33	0.03	–0.15	0.04	H	5.49E – 07	5.49E – 07	–2.090	T
LP738 – 43	18.08		0.56		He	2.82E – 06	5.79E – 05	–3.582	*
LP498 – 26	14.77	0.20	–0.14	0.20	He	5.70E – 06	5.48E – 04	–1.790	L
LP380 – 5	15.63		1.08		He	5.07E – 05	5.07E – 05	–4.080	*
LP856 – 53	15.20	0.02	0.38	0.03	H	3.06E – 05	1.01E – 03	–3.252	C
LP799 – 73	17.12	0.01	0.72	0.02	He	2.09E – 05	2.73E – 04	–3.784	C
G200 – 39	15.06	0.01	–0.04	0.03	He	8.14E – 07	8.16E – 06	–2.219	T
LP560 – 73	18.15		0.57		He	2.30E – 06	2.78E – 05	–3.597	T
LP686 – 32	17.10		0.69		He	3.10E – 06	1.58E – 05	–3.703	ref. 37
LP387 – 36	17.13	0.14	1.07	0.17	He	1.81E – 05	1.30E – 04	–4.073	T
LP70 – 172	17.57	0.09	0.87	0.15	H	1.34E – 05	1.40E – 04	–3.920	T
G206 – 17	16.44		0.40		H	4.39E – 06	8.04E – 05	–3.284	*
G206 – 18	17.07		0.57		H	4.15E – 06	3.15E – 05	–3.544	*
LP813 – 17	18.18	0.01	0.72	0.03	He	7.30E – 06	9.66E – 05	–3.784	C
VBs11	16.68		0.88		He	7.13E – 05	1.02E – 04	–3.933	ref. 39
L283 – 7	14.77	0.01	0.67	0.02	He	4.85E – 05	4.57E – 04	–3.728	C
L427 – 60	14.64	0.01	–0.02	0.02	He	9.74E – 06	6.25E – 04	–2.289	C
L573 – 108	14.44	0.01	0.04	0.02	He	1.99E – 05	1.45E – 03	–2.494	C
LP761 – 114	17.45	0.01	1.75	0.03	He	2.46E – 04	5.10E – 03	–4.439	C
LP577 – 71	14.42	0.01	0.56	0.02	H	2.48E – 05	1.66E – 04	–3.530	C

Columns from left to right; star identification, V-band magnitude and standard deviation, $V-I$ colour index and standard deviation, adopted atmospheric composition, raw (subscript r) and corrected (subscript c) $1/V_{\max}$ values for each star, luminosity of the star in solar units and the source of $V-I$ photometry (C is the Cerro Tololo Observatory, L is the Lowell Observatory, T is the McDonald Observatory in Texas and * is Leggett, personal communication). Following Liebert, Dahn and Monet⁷, we used a modification of the $1/V_{\max}$ method^{20,40} to compute the WDLF. This strategy takes into account the fact that each star's contribution to the luminosity function is a function of its proper motion, but a practical limit is reached when the sample volume is small enough that the probability of occupancy is $\lesssim 50\%$ (ref. 41). Nevertheless, in an attempt to achieve a volume-limited sample, stars are often excluded from WDLFs by imposing (high) proper motion and/or (bright) magnitude restrictions which are much more severe than the original survey limits. For example, for the carefully selected data in ref. 7, we calculate $\langle V/V_{\max} \rangle = 0.369 \pm 0.046$. But because this is $\sim 3\sigma$ from the value expected for a complete sample $\langle V/V_{\max} \rangle = 0.5$ (see ref. 20 for a good explanation), we conclude that the reported⁷ downturn in the WDLF is not well defined. Liebert, Dahn & Monet were fully aware of this possibility⁴² and only claimed that a shortfall of WD counts began to occur at $\log(L/L_\odot) \approx -4.4$. As expected, our sample of WDs is not complete: $\langle V/V_{\max} \rangle = 0.324 \pm 0.046$, but we have corrected for incompleteness as described in Fig. 1 legend.

opacities and neutrino rates, the current best estimates for the hydrogen and helium surface layer masses (fractional masses of 10^{-4} and $10^{-2} M_\odot$, respectively) and carbon–oxygen cores. We computed theoretical WDLFs assuming a standard infall star formation rate¹⁹ with Salpeter initial mass function, pre-WD evolutionary ages from evolutionary calculations² and a reasonable initial–final mass relation³.

The resulting theoretical WDLFs for disk ages spanning 7–15 Gyr are shown in Fig. 1. Each computed WDLF has been normalized to the observed space density by integrating over the

luminosity limits of the observed sample. The best fit to our observed WDLF (bar histogram) suggests an age for the local Galactic disk of $9.5^{+1.1}_{-0.8}$ Gyr (1σ , computed as in refs 7 and 20). Note that arbitrarily old models are consistent at the $+2\sigma$ level. Our age determination seems quite similar to the best previous estimate⁷ derived from the WDLF. However, when fitted with the isochrones shown in Fig. 1, the data of ref. 7 yield an age of $7.5^{+0.5}_{-0.5}$ Gyr (1σ), over 20% less than their original estimate.

It is unlikely that factors other than the age of the Galactic disk have created the more gradual downturn at the faint end of the

WDLF which we report here. In fact, the age of 9.5 Gyr derived here must be regarded as a robust lower limit as almost any imaginable systematic effect would increase the numbers of faint WDs detected (or equivalently lower the grid of theoretical curves), and would therefore increase the derived disk age. Even so, two possible systematic problems deserve attention: the dispersions in composition and mass among WDs.

As a test of the influence of assumed surface composition, we recomputed our observed WDLF twice, first assuming all hydrogen-rich and then all helium-rich atmospheres. The best fits resulting from these tests yield age estimates of ~ 13.0 Gyr and ~ 9.2 Gyr, respectively. We conclude that any future revisions to the assumed atmospheric compositions are more likely to increase rather than decrease our determination of the Galactic disk age.

With the photometric data alone, it is difficult to test whether WDs of significantly larger or smaller mass than implied by the $0.6 M_{\odot}$ assumed here affect our WDLF, particularly at the faint end on which the disk age estimate depends most. The discovery of ESO439–26, for which a trigonometric parallax and detailed atmosphere models give $\log(L/L_{\odot}) = -4.95$ (ref. 21), proved that at least one very low luminosity WD of high gravity and blue colour exists. Had this object been in our sample, our procedure would have placed it at $\log(L/L_{\odot}) = -4.2$ in Fig. 1 (two bins brighter than that determined from its trigonometric parallax) but the shape of the WDLF and age derived from it would change very little.

As an additional test of the effects of assumed mass, we recomputed the observed WDLF assuming two extreme choices for all stars, $0.5 M_{\odot}$ and $0.7 M_{\odot}$. The resulting ages derived from these two test cases were 9.2 and 9.7 Gyr, respectively, well within 1σ of the age derived from Fig. 1. In fact, all three objects in our faintest bin would have to be of exceptionally low mass to have created a (false) more gradual downturn in the WDLF than reported⁷. In view of the small and roughly symmetrical dispersion about the mean WD mass of $(0.590 \pm 0.137) M_{\odot}$ (1σ) (refs 22–24), it is unlikely that three WDs of unusually low mass (and no high-mass WDs) distort our WDLF unless strong selection effects favour the detection of cool, low-mass WDs (ref. 24). Moreover, our WDs were identified as possible non-physical pairs and/or unresolved composites whenever the components ΔV and $\Delta(V-I)$ gave discrepancies in the photometric parallax¹³. Such pairs were excluded from our sample, which effectively discriminated against WDs of unusually high or low mass. Nevertheless, trigonometric parallaxes are needed for these objects before this concern can be definitively addressed.

We conclude that any steep downturn in the WDLF is at least one bin fainter in luminosity than indicated by the observational samples published to date. It is interesting to speculate on the potential for discovering other cool WDs. Assuming our present sample size ($N = 50$) and the 9.5 Gyr curve, ~ 0.3 stars are expected at $\log(L/L_{\odot}) \approx -5.0$, hence it is not surprising this bin is currently empty. Our observation programme contains ~ 250 new spectroscopically identified WDs for which photometric parallaxes are currently being determined. We predict that the entire sample will yield ~ 15 additional stars in the $\log(L/L_{\odot}) = -4.5$ bin, but only 1–2 stars in the -5.0 bin. However, the probability of finding stars in this bin increases dramatically if the age of the Galactic disk is greater than ~ 9.5 Gyr. Should our continuing photometry result in the discovery of WDs in the $\log(L/L_{\odot}) \approx -5.0$ bin, then the ~ 14 – 17 Gyr ages of globular clusters would be strongly confirmed. However it is already clear from our sample of WDs that stellar remnants comprise a larger fraction of the dynamical mass of the Galaxy in the solar neighbourhood, and that the minimum age of the Galactic disk is substantially greater than suggested by previous studies of the WDLF.

Eggen, Lynden-Bell and Sandage²⁵ have argued that the Galaxy formed by rapid collapse from proto-galaxy to thin disk, although the timescale for the formation of the spheroidal component and globular cluster population is in dispute^{26–29}. Chemo-dynamic simulations of the formation process indicate that the timescale for complete collapse to thin disk and the onset of significant star formation could be as little as 1 Gyr or as long as 4–6 Gyr, depending upon how the star formation rate scales with disk surface density^{30,31}. Our interpretation of the WDLF is consistent with an emerging picture of the evolutionary history of the Galaxy, in which the halo formed ~ 14 – 17 Gyr ago^{8,9} and the bulge globular clusters formed ~ 12 – 14 Gyr ago¹⁰, with a modest hiatus before star formation commenced in the local disk ~ 10 Gyr ago.

A lower limit to the age of the Universe may be determined by adding the time span between the Big Bang and the formation of the Galaxy (~ 1 Gyr) to the Galactic disk age derived from the WDLF, plus any possible delay between the onset of star formation in the halo and local disk (≥ 1 Gyr, considering the above discussion). Our WDLF therefore suggests a firm minimum age of ~ 11.5 Gyr for the Universe, which is more than 2σ older than the current Hubble expansion age estimates of 8–9 Gyr (refs 8, 32–35) (which assume an inflationary universe in which $H_0 \approx 80 \text{ km s}^{-1}$ and $\Omega = 1$). $\square$

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Lasing from conjugated-polymer microcavities

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FOLLOWING the discovery¹ of electroluminescence in poly(*p*-phenylenevinylene) (PPV), considerable effort has been directed towards the realization of optoelectronic devices based on semi-conducting conjugated polymers of this type^{2–6}. But the viability of these materials for such applications depends critically on the nature of the photoexcited states—in particular, whether they are predominantly non-emitting interchain species^{7–9} or emitting intrachain species¹⁰. One way to study this fundamental issue is in a device structure known as a microcavity¹¹, which offers the possibility of using quantum electrodynamic effects to alter (and hence probe the nature of) spontaneous and stimulated emission in these materials^{12–17}. Here we make use of such a structure to demonstrate optically driven laser activity in devices based on solid films of PPV. This demonstration of lasing provides direct support for a model¹⁰ in which the main photoexcitation in PPV is an emissive intrachain species, and opens the possibility of electrically driven polymer-based lasers.

In order to achieve lasing it is advantageous to use a cavity with high quality factor Q , and dimension of the order of the wavelength of the light emitted. The active material in our devices is a film of the polymer PPV (Fig. 1). We chose PPV as it is relatively robust, can show a high photoluminescence efficiency¹⁸ and, in spite of indications to the contrary^{7,8,19}, it can exhibit gain in pump-probe experiments with ultraviolet excitation, as we show later. As the enhancement of radiation by a microcavity is a strong function of Q we fabricated cavities with highly reflecting mirrors. As the bottom mirror we used a commercially available, ~100-nm-thick distributed Bragg reflector (DBR), which is a stack of alternating layers of high and low refractive indices, with better than 99% reflectivity at visible wavelengths (Comar Limited). To cover the entire visible range, the DBR mirror is made of various stacks with different layer thicknesses (chirped DBR), thus longer wavelengths are reflected deeper inside the stack. This type of mirror causes the cavity length to increase in discrete steps as the wavelength is increased. A virtue of this structure is that, despite the fact that the cavity has a dimension of the order of the wavelength, several discrete modes can still be supported. On top of the DBR we spin-coated a layer of the tetrahydrathiophene

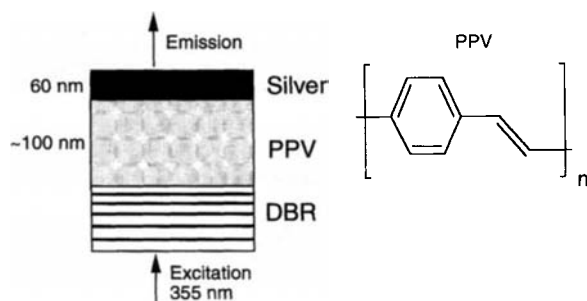


FIG. 1 Structure of the microcavity used. We used different batches of PPV which were converted at various temperatures ($\geq 200^\circ\text{C}$) in a vacuum (batch A: bromide, 4 hours conversion; batch B: chloride, 10 hours conversion) to yield PPV films with thicknesses around 100 nm. Depending on the temperature, the films were either substantially or fully conjugated and the solid-state photoluminescence efficiency was measured to be as high as 80% for batch A (using an integrating sphere with 457 nm excitation). The temperature range used in the conversion process had no effect on achieving lasing.

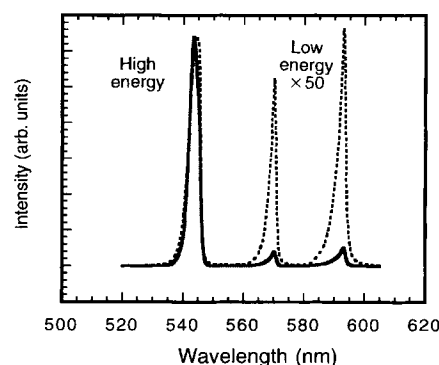


FIG. 2 Emission spectrum of the PPV microcavity structure at two excitation energies, 0.05 μJ and 1.1 μJ . The light was collected from the device, with a 16.5° emission cone (full angle). PPV from batch A was used for this device (see Fig. 1 legend).

halide precursor to PPV¹. The 60-nm-thick top mirror was formed by the thermal evaporation of silver. Extreme caution was taken to avoid any oxidation of the PPV film¹⁰, and all the above steps were carried out within a dry-nitrogen-filled glovebox. Several devices were made from different batches of PPV.

The devices were excited by the frequency-tripled output of a regeneratively-amplified Nd:YAG laser, consisting of pulses of 200–300 ps, with a repetition rate of 1 kHz and a centre wavelength of 355 nm. The beam was focused to a diameter of 250 μm on the sample and the maximum incident pulse energy was 5 μJ . The sample was excited through the DBR which had 30% transmission at 355 nm. After leaving the sample chamber, the emission was immediately collimated and passed through an adjustable iris which could be set to control the measured emission cone from the device. The photoluminescence spectra were recorded using a spectrograph and a CCD (charge-coupled device) camera.

The measured full width at half maximum (FWHM) of the cavity mode, measured with a 0.8° acceptance cone (full angle), was 1 nm which is close to the 0.65 nm response of the spectrograph, indicating the high Q of the cavity. When the light was collected from a 16.5° cone, the FWHM was ~ 4 nm. Figure 2 shows the measured photoluminescence spectra for excitation energies (inside the cavity) of 0.05 μJ and 1.1 μJ . At the lower energy, emission is distributed almost equally between three cavity modes that arise from the structure of the DBR mirror. Note that the modes are not equally distributed in energy as their spacing is mainly determined by the wavelength-dependent effective length of the DBR mirror. The dramatic change in the photoluminescence spectrum, with the mode at the PPV gain peak wavelength (545 nm) dominating the emission at high output powers, indicates that the emission is mainly stimulated and hence that the device is lasing. If there were no gain (stimulated emission), the spectrum would just scale with power and no mode would dominate. We also notice that the two additional modes continue to have the same relative power, which is consistent with the presence of gain in the main mode only. These results suggest that by introducing extra modes, the determination of the lasing phenomenon in a wavelength-scale device becomes straightforward.

Figure 3a shows the output power of two devices as a function of input power. This figure does not show any abrupt change in the output power (the usual definition of lasing threshold) and this may suggest a high coupling efficiency of spontaneous emission into the lasing mode. In such cases a different criterion for the lasing threshold may be invoked¹¹—it is defined as the point at which spontaneous and stimulated emission powers are equal. Figure 3b shows the ratio between the integrated power under the lasing mode and the power emitted across the rest of the spectrum. For low powers, where the emission is spontaneous, the ratio for

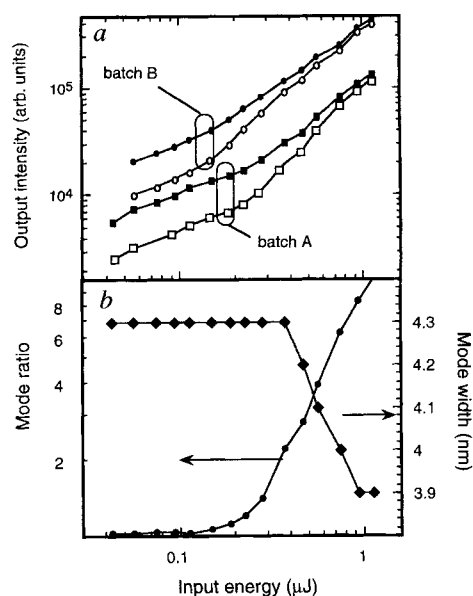


FIG. 3 *a*, Output power as a function of input energy for two microcavity devices made from different PPV batches (Fig. 1 legend). The filled points denote the power integrated between 500 nm and 600 nm and the open points are for the power under the main mode only. *b*, The ratio of the power within the main mode to that in the rest of the spectrum as a function of input energy (filled circles). When this ratio equals two, spontaneous and stimulated emission rates are equal. Also shown is the energy-dependent linewidth of the main mode (filled diamonds). PPV from batch A was used for this device.

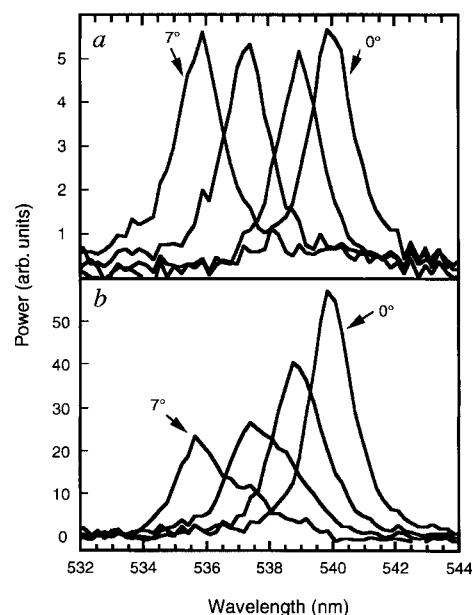


FIG. 4 Output spectra for different viewing angles between 0° (normal direction) and 7°. *a*, Low output power (below lasing threshold). *b*, High output power (above lasing threshold). The increased directionality for high powers is shown by the decrease of peak height with increasing angle. The emission was measured from a 0.8° cone. The curves were shifted horizontally to allow easier comparison between the two subfigures. The measured shift, in the peak wavelength, between the two extreme angles was less than 1 nm.

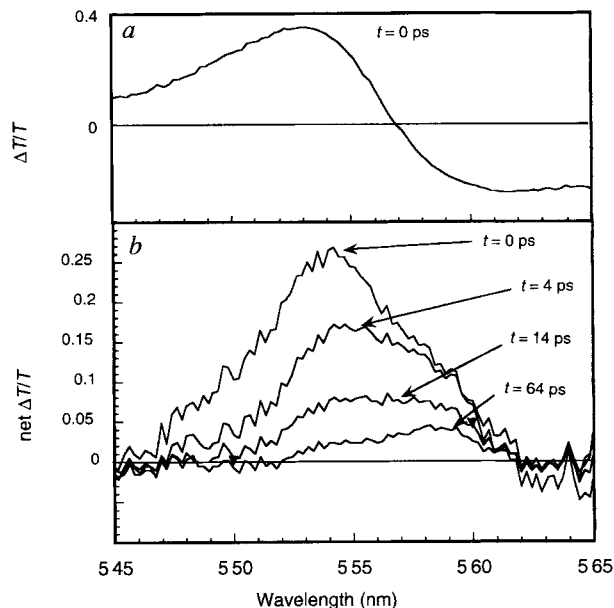


FIG. 5 *a*, Spectrally resolved transient changes in optical transmission, $\Delta T/T$, through a PPV microcavity at delay $t = 0$ after the excitation pulse (at 325 nm). *b*, The same data, shown for a range of delays, after correction for the modulation of the optical cavity length. This gives the net $\Delta T/T$, showing the presence of stimulated emission (gain). The presence of regenerative amplification makes the peak position of the gain follow that of the cavity mode and hence, it also shifts with time. The microcavity was formed with top and bottom silver mirrors (35 nm and 45 nm respectively) sandwiching a ~ 100 nm film of PPV. The transmission of the device at the resonance wavelength was 2% (the DBR-silver devices had a transmission below the measurement sensitivity). The mode of the cavity was positioned at ~ 555 nm, close to the PPV peak emission, and had a linewidth of ~ 10 nm. PPV from batch B was used for this device.

these devices is close to unity. At higher powers, stimulated emission is only present in the lasing mode, and hence this ratio increases. This increase in the relative power within the main mode was also seen in structures that exhibited an additional mode at a shorter wavelength than the main mode. Hence, the ratio change is due to gain in the main mode and not photo-induced absorption which occurs at longer wavelengths⁹. We note that the lasing threshold is reached when this ratio doubles. Also shown in Fig. 3*b* is the power-dependent FWHM of the main mode. Note that the linewidth starts to narrow at the same point, where the mode ratio equals two. The transition from multi-mode to single-mode operation and the narrowing of the linewidth both indicate increased coherence, as expected from a lasing device. Figure 4 shows the angular dependence of the emission spectrum at two output powers (above and below the lasing threshold). The increase in the directionality of emission at high output power (Fig. 4*b*) is further evidence that the device is lasing.

The demonstration of lasing using a solid film of a conjugated polymer also allows us to shed light on a fundamental question related to this type of material. It has been claimed that most of the photoexcited states in this type of polymer are non-emissive interchain polaron pairs and that these are formed instantaneously⁷⁻⁹. This non-emissive species was shown to be responsible for photoinduced absorption which masked the gain produced by intrachain excitons⁷⁻⁹. If a large number of the interchain species are formed at time $t = 0$, one would not expect lasing even in a microcavity structure. This is both because the microcavity has no effect on the nature of photoexcited states, only on their radiative decay rate, and also because the excited state absorption in the emissive region of the spectrum prevents gain in the cavity.

We find that when the polymer is treated carefully during both synthesis and device preparation, pump-probe measurements at short times after excitation can demonstrate gain, even with ultraviolet excitation. We show this for PPV in a microcavity structure, with silver top and bottom mirrors. These measurements were carried out using the time-resolved differential transmission technique with 150 fs pump pulses at 325 nm (ref. 20). In

this technique, the fractional change ($\Delta T/T$) in the transmission (T) of a broad-band (white-light) probe in the presence of the pump is spectrally resolved and measured as a function of the time delay between the pump and probe pulses. For this cavity structure, effects are due to both the excited state emission and absorption in the PPV, and the modulation of the cavity length as a result of the excited state population (described by the resonant third-order nonlinear optical susceptibility). The latter is evidently a response that follows the differential of the cavity mode function with respect to wavelength, as shown in Fig. 5a. This feature results from the relation $\Delta T = T(\lambda_0 + \Delta\lambda) - T(\lambda_0)$ where λ_0 is the wavelength of the mode and $\Delta\lambda$ is the mode shift. Figure 5b shows the probe amplification after correction for the effects of the cavity length modulation. Note the high values at $t = 0$ (>25%) that result from the enhancement of the stimulated

emission by the cavity (regenerative amplification). Also seen in this figure is the dynamic shift of the mode peak, with significant positive gain present until 64 ps. The results shown in Fig. 5 are fully consistent with the demonstration of lasing, shown earlier, and taken together these provide very direct support for the model in which the main photoexcitation in high-quality PPV is the intrachain exciton¹⁰.

We have demonstrated lasing in a high- Q microcavity device based on PPV. This has the structure appropriate for electrical pumping (in contrast to structures which use conjugated polymers in solid or liquid solutions^{21,22}), although much remains to be done to improve the ease with which charge can be injected into the PPV. We conclude nevertheless that the quality of present polymers has reached a point where they can be considered as electrically pumped lasers for highly demanding device applications. □

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A chemical-detecting system based on a cross-reactive optical sensor array

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THE vertebrate olfactory system has long been recognized for its extraordinary sensitivity and selectivity for odours. Chemical sensors have been developed recently that are based on analogous distributed sensing properties^{1–4}, but although an association between artificial devices and the olfactory system has been made explicit in some previous studies^{4,5}, none has incorporated comparable mechanisms into the mode of detection. Here we describe a multi-analyte fibre-optic sensor modelled directly on the olfactory system, in the sense that complex, time-dependent signals from an array of sensors provide a 'signature' of each analyte. In our system, polymer-immobilized dye molecules on the fibre tips give different fluorescent response patterns (including spectral shifts, intensity changes, spectral shape variations⁶ and temporal responses) on exposure to organic vapours, depending on the physical and chemical nature (for example, polarity, shape and size) of both the vapour and the polymer. We use video images of temporal responses of the multi-fibre tip as the input signals to train a neural network for vapour recognition. The system is able to identify individual vapours at different concentrations with great accuracy. 'Artificial noses' such as this should have wide

potential application, most notably in environmental and medical monitoring.

A critical problem solved by the sense of smell is how to incorporate into a single system both selectivity for many disparate chemicals (broad-band response) and high sensitivity. One way to achieve this would be to have a multitude of specific receptors with high binding coefficients, one for each molecule of interest to the organism. There is little experimental evidence for this, with the exception of pheromone detection in insects where a specific ligand/receptor/neural pathway seems to exist³¹. In vertebrates, however, physiological, anatomical, biochemical and lesion studies all support the view that the distinctive molecular signatures of odour compounds are encoded by cross-reactive receptor cells, in conjunction with neuronal integrative circuits, by the generation of response patterns that are widely distributed in space and time⁷. Using similar spatio-temporal patterning, we have incorporated a number of these biological properties into an odour sensing device. As in previously developed systems^{2,4,8–13}, this device does not rely on specifically selective sensors. The crucial element of such systems is to capture information about the analyte molecule in a distributed fashion that is encoded sufficiently to allow discrimination from other closely related chemical structures.

In an effort to implement this concept of distributed detection, we have incorporated an array format into the construction of the sensor, using a bundle of 19 individual multi-mode optical fibres. In recent years, the array approach has also been taken by others in the development of vapour sensors based on surface-acoustic-wave (SAW)^{2,8,9}, electrochemical¹⁰, conducting polymer^{4,11} and piezoelectric^{12,13} methods. Conductive polypyrroles, for example, are now being used in commercial devices to identify odours based on the conductivity changes that occur as analytes adsorb onto the sensor surface. Acoustic-wave-based sensors, on the other hand, measure frequency deviations associated with the slight mass changes induced by vapour molecules adsorbing onto oscillating, polymer-coated quartz sensors. Because each of the current

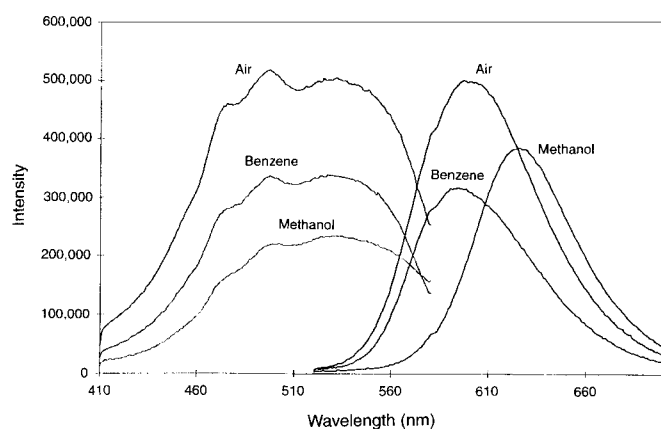


FIG. 1 Fluorescence spectral changes of polymer-immobilized Nile Red with polarity changes imposed by different vapours (excitation spectra on left, emission spectra on right).

METHODS. The distal end of a single-core, multi-mode optical fibre was polished, functionalized, immersed in a solution of PS802/MMA/Nile Red (53% PS802, (80–85%) dimethyl (15–20%) (acryloxypropyl)methylsiloxane copolymer; 3% methylmethacrylate; 44% Nile Red (1 mg per ml in chloroform)) containing 30 mg per ml initiator benzoin ethyl ether (BEE), and illuminated with ultraviolet light (330 nm) for 20 s to form a 100- μ m layer at the fibre tip. Both excitation and emission scans were acquired first with the fibre in air, then with it exposed to saturated benzene and methanol vapours via insertion into the headspace of vials containing the individual solvents.

approaches involves the observation of a single parameter (signal intensity), expanding the analyte base requires significant increases in the number of sensors in the array. Optical techniques, however, have the ability to provide many kinds of complex information simultaneously, including (but not limited to) changes in intensity, fluorescence lifetime, wavelength and spectral shape, thereby offering a potentially attractive alternative to existing single event-monitoring methods. Increasing the dimensionality of a system by observing many different parameters simultaneously should make it possible to produce a more sensitive, multi-analyte-detecting device with fewer sensors. Though several optical techniques have been explored for vapour sensing^{14–19}, to date there have been no reports of fibre-optic arrays being used in a broad-band chemical sensing tool of this nature.

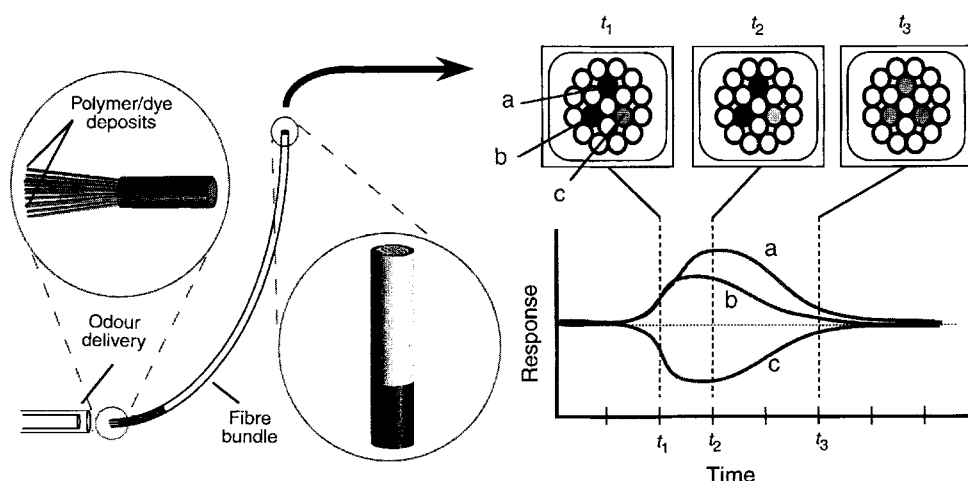
Here we use a single solvatochromic dye (Nile Red) immobilized within different polymer matrices, each having a different baseline polarity. Nile Red exhibits large shifts in its emission wavelength maximum (Fig. 1) with changes in local polarity, and has been employed as a fluorescent probe in biological applications^{20–22} and solvent polarity studies^{23,24}. (See ref. 6 for a detailed discussion of how fluorophores can be influenced by environ-

ment). By immobilizing Nile Red in polymer matrices of varying polarity, hydrophobicity, pore size, flexibility and swelling tendency, we create unique sensing regions that interact differently with molecules of individual vapours, giving rise to different fluorescence responses when exposed to organic vapours.

The primary sensors in the olfactory system are millions of sensory receptor neurons lining the nasal cavity. On stimulation with an odorant consisting of a single molecular species, these cells respond with a variety of amplitudes, latencies, and time courses²⁵. It is also known that cells in the next relay, the olfactory bulb, respond with various temporal patterns^{26,27} initiated by the sniffing process. These temporal responses appear to be crucial to the odorant encoding process. In the device described here, presentation of a single compound to the array generates changes in fluorescence with different amplitudes and time courses in each of the different fibre sensors, triggered by the onset of the analyte pulse. Data are gathered by monitoring the fluorescence of each sensor in the array as pulses of different vapours are applied (Fig. 2). For example, in response to a two-second pulse of benzene vapour, some fibres exhibited a large, rapid decrease in fluorescence while others showed smaller, more gradual decreases (Fig. 3a).

FIG. 2 A schematic representation of the odour-sensing process. Vacuum-controlled pulses of various air/solvent vapour dilutions are delivered directly to the distal ends of fibres in the sensor bundle. Exposure to vapour induces changes in fluorescence intensity at a given wavelength which are then recorded and plotted versus time to produce a temporal response pattern for each sensing site to a given odour. In this illustration, response profiles for three arbitrary sensors (a, b and c) in the array are shown, along with three representative video frames at different times (t_1 , t_2 and t_3) along the curves.

METHODS. The array comprises of 19 single-core, 300- μ m-diameter fibres packed together and enclosed at the proximal ends in a stainless-steel sheath. The distal ends are individually coated with different polymer/dye combinations via either of two methods: solvent evaporation dip-coating or photopolymerization. In the first method, silanized fibre tips are repeatedly dipped in prepared Nile Red/polymer solutions in chloroform, allowing the solvent to evaporate between additions. The second technique employs through-fibre propagation of ultraviolet light (see Fig. 1 legend) to polymerize dye/monomer/initiator solutions at the fibre tip. Both approaches result in relatively even polymer layers, with Nile Red dye molecules trapped within the matrix. Layer thickness can be controlled by the number of dips or length of ultraviolet exposure, respectively. The completed sensor is then incorporated into an illumination and



detection system that uses filter wheels, shutters and dichroic mirrors to select and direct light of the appropriate wavelength (excitation, 535 nm; emission, 610 nm). Light is both delivered to, and collected from, the proximal end of the fibre bundle. Fluorescent images of the array are projected onto a CCD camera and captured with a video acquisition system. A computer-controlled analyte delivery system (updated from a version previously described²⁹) delivers pulses to the array having carefully controlled onsets, rise times, plateaus and falling phases. Fluorescence response is then plotted versus time to produce a series of patterns that are introduced as inputs to the neural processing system.

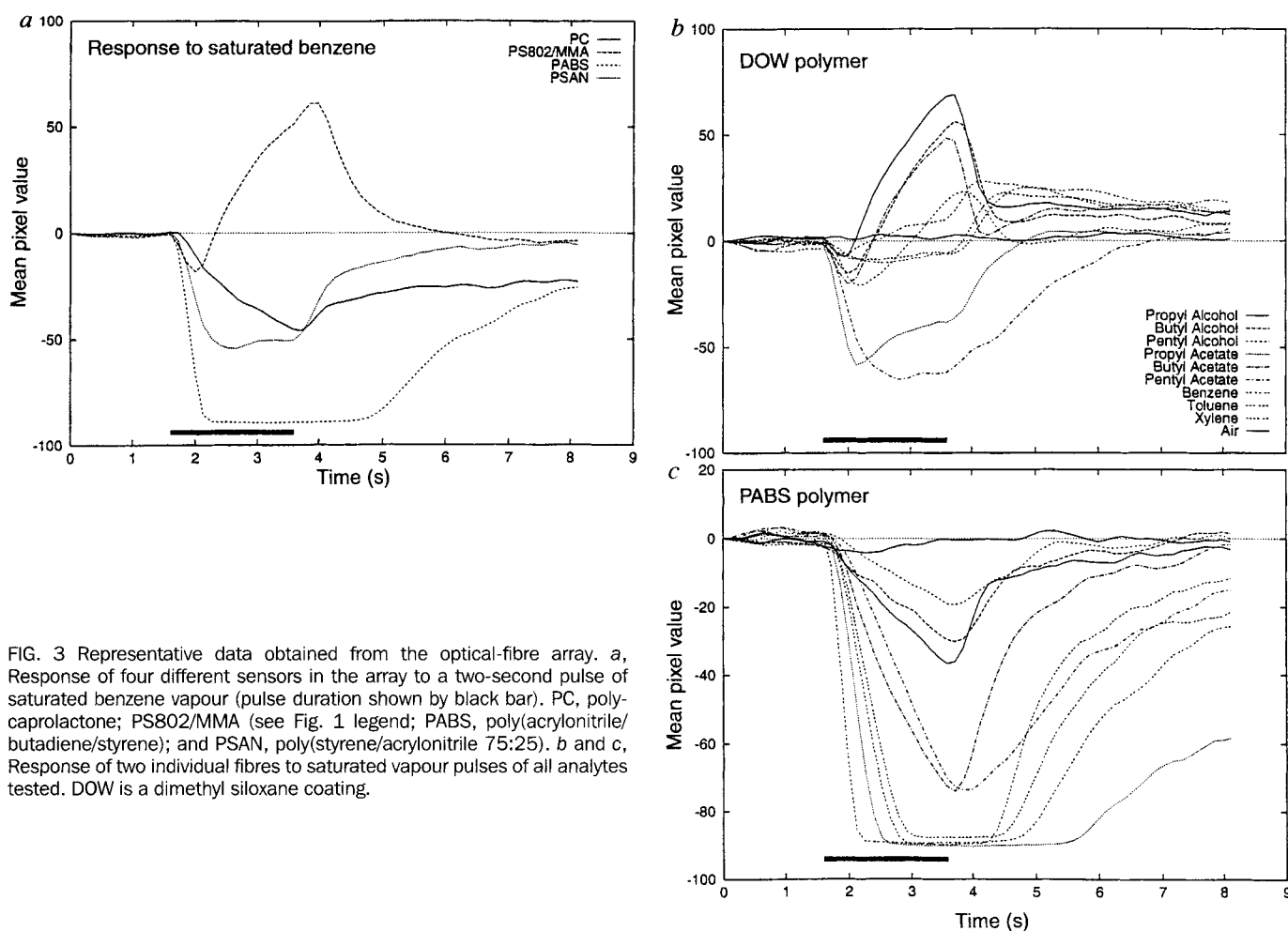


FIG. 3 Representative data obtained from the optical-fibre array. *a*, Response of four different sensors in the array to a two-second pulse of saturated benzene vapour (pulse duration shown by black bar). PC, polycaprolactone; PS802/MMA (see Fig. 1 legend); PABS, poly(acrylonitrile/butadiene/styrene); and PSAN, poly(styrene/acrylonitrile 75:25). *b* and *c*, Response of two individual fibres to saturated vapour pulses of all analytes tested. DOW is a dimethyl siloxane coating.

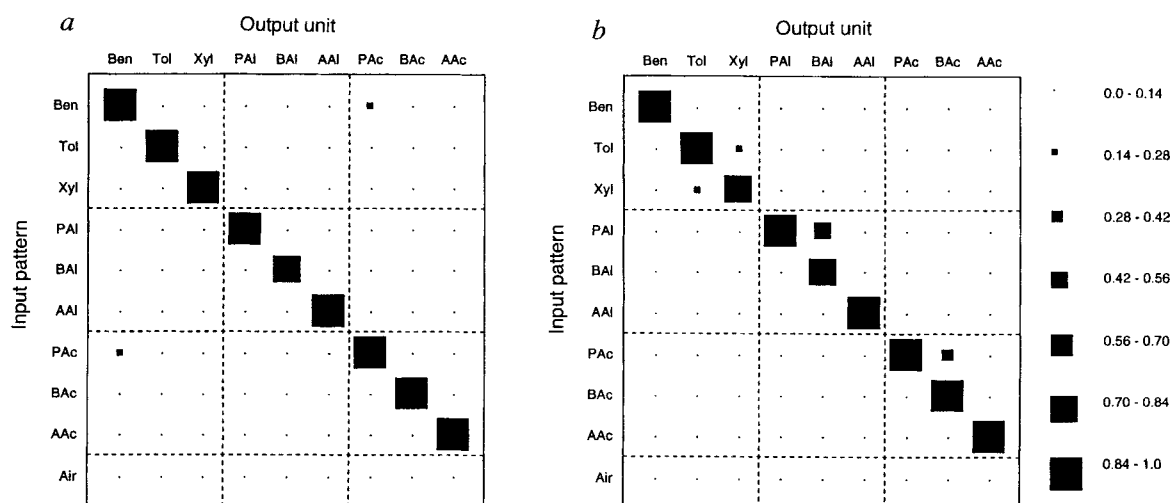


FIG. 4 Neural-network performance diagrams for a range of aromatic, alcohol and acetate vapours at two different concentrations; saturated vapour (*a*) and 1:1 diluted in air (*b*). The network is trained with the sensor-array responses to each of the vapours along the vertical axis. The final system is then tested with a new set of data, and the output is listed along the horizontal axis. The size of the filled squares indicates the degree of confidence with which the network has made a particular identification (see key at right of figure). Ben, Benzene; Tol, Toluene; Xyl, Xylene; PAI, Propyl Alcohol; BAI, Butyl Alcohol; AAI, Amyl (Pentyl) Alcohol; PAc, Propyl Acetate; BAc, Butyl Acetate; AAc, Amyl Acetate.

METHODS. A number of neural-network configurations were investigated using the Stuttgart Neural Network Simulator, version 3.2³⁰. The results shown here are from a feed-forward network of 100 input units (10 sequential time-points from each of 10 fibre sensors), 10 hidden units, and 9 output units that was trained using the back-propagation with momentum learning algorithm. The network was trained with data from four replicates until a minimum error criterion of 1.0 was reached (~300 iterations), then tested with separate data from a fifth replicate.

Response differences in the overall shape and termination of the curves were also observed.

Analogous to individual sensory receptor cells in the olfactory system, individual sensors in the fibre array displayed different response profiles when tested with different analytes, as can be seen by comparing two sensors of the array (Fig. 3b and c). In this way, different temporal patterns were generated over the entire fibre array for each analyte. Responses of the sensors were found to remain stable for several weeks, over many hundreds of analyte applications.

Both the reproducibility and variety of sensor responses to different analyte structures suggested that sufficient information for analyte recognition was present in the overall array response. To test this hypothesis, a variety of neural networks were investigated for their ability to identify analyte structure correctly. Both two- and three-layer feed-forward networks were evaluated with different numbers of hidden and output units. Networks were trained with data from four consecutive trials and then tested with a different data set generated in a subsequent trial. Neural networks using temporal-response profiles demonstrated a marked improvement in analyte recognition ability over networks using only integrated data (generated by calculating the areas under the response curves). The outputs of an optimized network trained with temporal data are given in Fig. 4. As can be seen from this figure, at both analyte concentrations, the network correctly identified all analytes. If integrated fluorescent signals were used instead of the temporal signals, the error rate increased markedly.

This type of sensor may ultimately be useful in a diverse range of applications for medical diagnostics, environmental monitoring, and product quality control. Current work is focusing on improving sensor sensitivity and detection limits, expanding the database of test analytes and exploring processing methods that provide information on analytes or concentrations that were not included in the training set. Preliminary data suggest that the device is highly effective in identifying components of mixtures, as well as characterizing analytes on the basis of their chemical characteristics, such as functional groups and relative molecular weight²⁸.

Finally, because the device has been designed to incorporate principles underlying olfactory function, it is possible that further investigation of the 'artificial' nose may provide insight into the functioning of the 'real' nose. □

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Widespread increases in low-frequency variability of precipitation over the past century

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PRECIPITATION (rain and snow) provides, through its participation in the global hydrological and energy cycles, most of the heat flux within the atmosphere. For this reason, a knowledge of precipitation variability is important to understanding the behaviour and changes of the Earth's climate system. Precipitation measurements have been made at thousands of sites over the past hundred years or so, and recent efforts have resulted in the compilation of global data sets^{1–3} and regional networks^{4,17}. The latter have been analysed to reveal high-frequency (up to inter-annual) regional trends in precipitation variability over the past century⁴. Here I exploit the global data sets to examine the low-frequency (decadal to multi-decadal) variability of precipitation over a similar time period. In agreement with other analyses⁵, I find that the global mean precipitation has not changed. The fluctuations about the mean, however, have increased significantly

(on decadal to multi-decadal timescales). That is, over the past century—during which climate warming has occurred—the global precipitation field has undergone changes on those scales, in which extremes have become more probable. This result is consistent with predictions from model simulations of global climate-warming scenarios^{6–8}.

Changes in the hydrological cycle are likely to affect regional and global precipitation patterns⁹. Such changes may have dramatic social and economic effects⁶. It then becomes imperative to study the effect of a climate-warming scenario—increasing CO₂ concentrations and rising global temperature—on precipitation. One way to study these effects is through general circulation models (GCMs), but these do not provide an unequivocal picture or high confidence of how precipitation might be affected^{9,10}. A more direct approach is to examine the records of precipitation measurements from around the globe. Karl *et al.*⁴ have considered daily precipitation totals at many stations over several regions around the globe and aggregated them into five categories of precipitation ranging from light to extreme. They then calculated the proportion of the seasonal and annual total falling in each category for several regions. By evaluating the significance of the regression of the percentage anomaly of precipitation total in each category with time, they showed that the high-frequency (up to interannual) variability of precipitation has increased over the United States in the past century. Here I extend the investigation in order to address the question of lower-frequency variability in the global precipitation field by employing a different strategy specifically designed to reveal low-frequency features. For this purpose I used the comprehensive precipitation data set for global

land areas¹⁻³. This data set includes monthly precipitation amounts from a total of 5,328 stations around the globe from the mid-1800s to the late 1980s. Most of the records are not continuous in time and the number of stations has varied.

The precipitation data exhibit high spatial and temporal variability that makes identification of trends from direct analyses of the time-series difficult. Modelling with the gamma distribution provides an alternative to detecting signals from the actual data. It fits precipitation data very well^{1,4,11} and allows the detection of changes in the precipitation field by monitoring changes in the less-variable parameters of the distribution. The gamma distribution is given by

$$f(x) = \beta^{-\gamma} x^{\gamma-1} e^{-(x/\beta)} / \Gamma(\gamma) \quad \beta > 0, \gamma > 0$$

where x is the random variable, Γ is the gamma function, and β and γ are the scale and shape parameters, respectively. According to Thom¹² the maximum likelihood estimates for β and γ from a sample of N measurements are given by

$$\hat{\gamma} = \left(1 + \sqrt{1 + 4A/3}\right) / 4A \quad \text{and} \quad \hat{\beta} = \bar{x} / \hat{\gamma}$$

where $A = \ln \bar{x} - (\sum \ln x) / N$, and $\bar{x}$ is the sample mean. For small sample sizes N , confidence intervals on θ (which can be either β or γ) are given by the statistic $T(\theta)$ defined as¹³

$$T(\theta) = 1 / \sqrt{I[d \log L / d\theta - (k_3(J) / 6I^2)((d \log L / d\theta)^2 - I)]} \quad (1)$$

where $\log L = -N\gamma \log \beta - N \log \Gamma(\gamma) + (\gamma - 1) \sum \log x - 1/\beta \sum x$, $I = E(d \log L / d\theta)^2$, $k_3(J) = 3dI/d\theta + 2E(d^3 \log L / d\theta^3)$, $E(\cdot)$ indicates the expected value and $d^n/d\theta^n$ the n th derivative with respect to θ . The distribution of $T(\theta)$ is $N(0, 1)$. Equation (1) suggests little error in the estimation of θ for N greater than about 30. This is also suggested from computer simulation with the gamma distribution¹⁴. Based on the above I present results on the temporal changes of the gamma distribution. Figure 1a shows values of $\hat{\beta}$ (dashed line) and $\hat{\gamma}$ (solid line) for Milwaukee station calculated from yearly totals in various 40-year intervals. From this figure it may be seen that, depending on the 40-year interval, $\hat{\gamma}$ may take on values in the range 27.0–48.0 and $\hat{\beta}$ may take on values in the range 1.30–2.25. Most importantly, this figure suggests the existence of trends in the parameters of the distribution, as both $\hat{\beta}$ and $\hat{\gamma}$ seem to be exhibiting a trend after the 1890s (positive for $\hat{\beta}$ and negative for $\hat{\gamma}$). It is worth pointing out that the existence of such trends may invalidate previous procedures for estimating global means based on gamma distributions whose parameters are estimated over a fixed 40-year period as they will not correctly represent precipitation amounts in some other 40-year period (as, for example, in refs 1,2). Figure 1b indicates that the mean ($\hat{\beta}\hat{\gamma}$) of the distribution remains virtually unchanged whereas the variance ($\hat{\beta}^2\hat{\gamma}$) increases. For example, the average of the first three values of $\hat{\beta}^2\hat{\gamma}$ is 91.3 whereas the average of the last three values is 134.8. This represents a significant increase in variance of 47.6% which appears to be associated with some kind of a jump (or low-frequency oscillation) rather than a gradual increase.

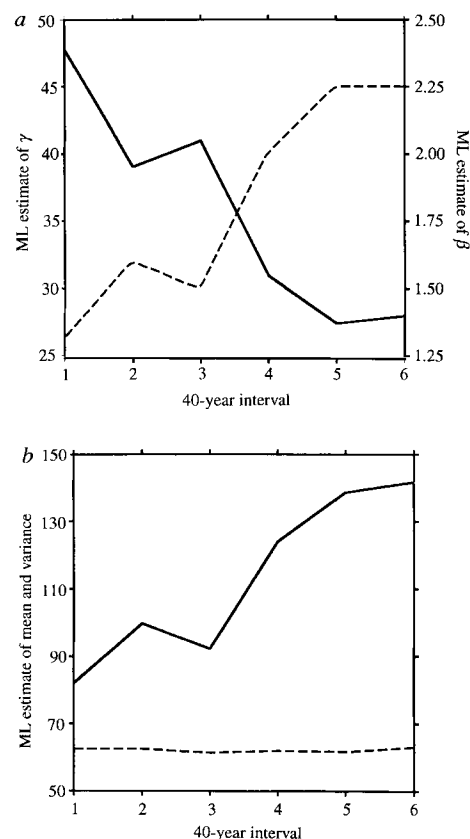


FIG. 1 a, Maximum-likelihood (ML) estimates of the parameters γ (left axis, solid line) and β (right axis, dashed line) of the gamma distribution for Milwaukee from annual precipitation totals in various 40-year time intervals. The number 6 corresponds to a window including the last 40 years of the station, 5 includes the 40 years when the window is moved by 10 years and so on. Such a strategy may by design enhance low-frequency variations. It considers a long enough non-overlapping period between the 40-year intervals, thus allowing for significant changes in the parameters of the gamma distribution. This figure indicates that γ exhibits a negative trend and β a positive trend. b, Maximum-likelihood estimates for the mean ($\beta\gamma$) (dashed line) and variance ($\beta^2\gamma$) (solid line). The units are cm for the mean and cm^2 for the variance.

The question is then whether such behaviour has been observed elsewhere on the globe. To assess this possibility rigorously, stations are needed with at least 90 years of data (this length provides the required sample size for an analysis similar to that in Fig. 1 to be performed). To avoid problems with incomplete records, only stations with complete monthly records over those 90 years can be included in the analysis. I considered the period

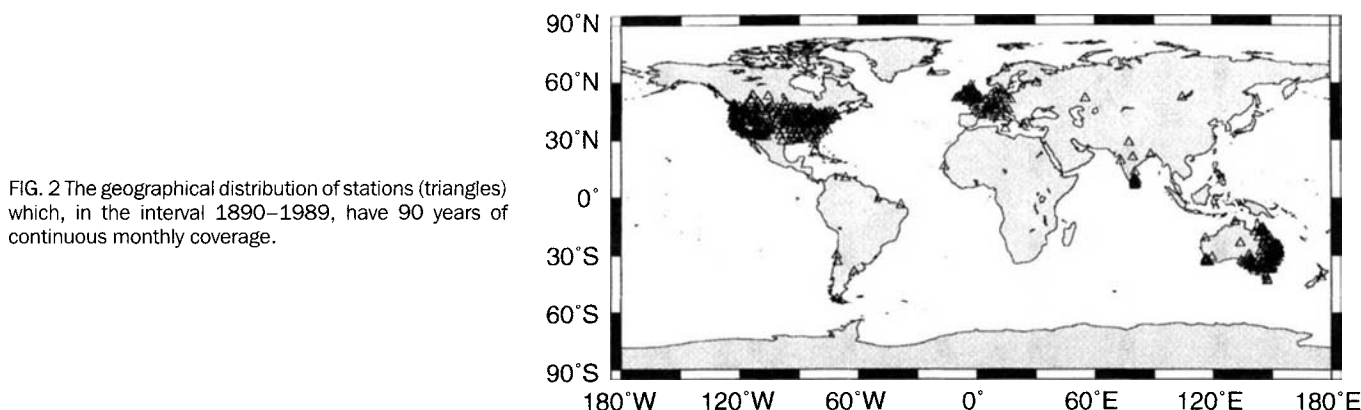


FIG. 2 The geographical distribution of stations (triangles) which, in the interval 1890–1989, have 90 years of continuous monthly coverage.

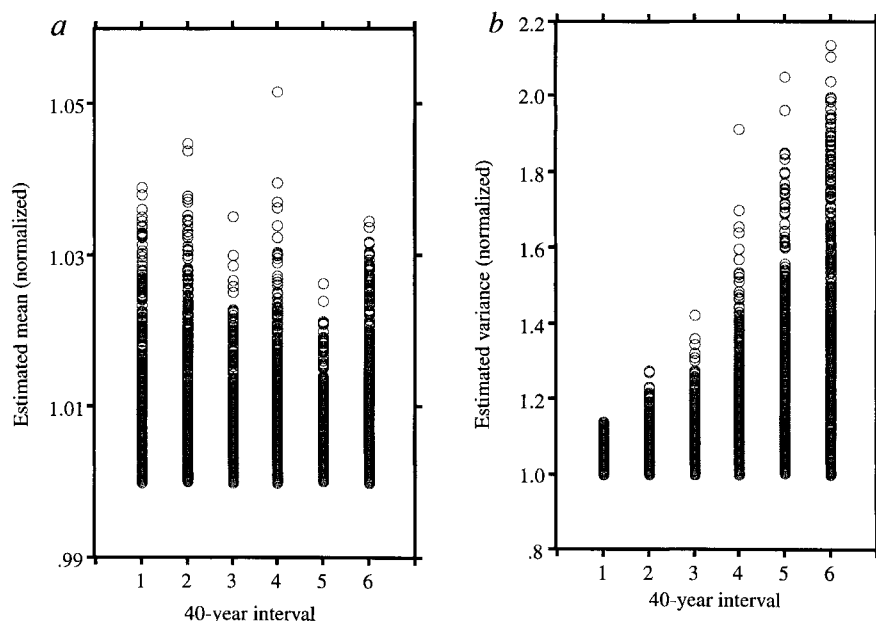


FIG. 3 Maximum-likelihood estimates of the mean ($\hat{\beta}_1$; a) and variance ($\hat{\beta}_2$; b) from all stations as a function of the 40-year interval. According to this figure the mean global precipitation exhibits no significant overall trend but the variance does.

1890–1989 (a period characterized by a marked positive trend in global temperature) and found that 411 stations—mostly in the United States, Europe and Australia (Fig. 2)—satisfy the above constraints. This highly non-uniform distribution and clustering of the stations prevents me at present from investigating the spatial features of low-frequency variability, but meaningful insights can be provided by repeating, for each available station, the procedure outlined in Fig. 1. Figure 3 shows $\hat{\beta}_1$ (panel a) and $\hat{\beta}_2$ (panel b) from all stations as a function of the 40-year interval. In order to avoid contaminating possible signals in this figure, due to widely varying precipitation climatologies over the globe, the values of $\hat{\beta}_1$ and $\hat{\beta}_2$ were normalized by their minimum of the six possible values. Such a normalization does not alter trends in $\hat{\beta}_1$ or $\hat{\beta}_2$, which is what I am looking for here.

Figure 3a suggests that overall the mean global precipitation has not changed over the past century. This result is consistent with the latest IPCC report⁵, in which, although certain regional trends are identified, it is concluded that global land precipitation during the twentieth century shows no significant trend. Among these regional trends are those of North America from 45° to 55° N (positive trend), of contiguous USA (weak positive trend), of northern Europe, north of 55° N (weak positive trend), of southern Europe (weak negative trend), and of eastern Australia (negative trend). Even though differences between the data sets and the statistical approach employed here prevents direct comparisons, my analysis indicates similar regional trends. These trends, in a composite graph like Fig. 3a, are embedded in the global picture of an overall zero trend.

On the other hand, Fig. 3b strongly indicates that an overall positive trend in precipitation variability has occurred. Analysis of surrogate data (that is, random data having similar statistical properties¹⁵ to the rainfall records) indicates that this trend is

statistically significant at a confidence level of 99%. More importantly, a careful examination of this figure reveals that, to a large extent, the overall trend is not due to a gradual increase, but rather is due to a pronounced change for 40-year intervals greater than 3 (see Fig. 1 legend for explanation of this nomenclature) where the variance becomes clearly larger than before. This is reflected in the fact that the overall trend is greater by almost a factor of two compared to the two sub-trends (for 40-year intervals 1–3 and 4–6, respectively). Remarkably, this 'jump' happens around the time a similar behaviour is visible in global temperature records (later 1920s). Global temperature records like the IPCC record¹⁶ display an overall positive trend characterized by a plateau up to about 1920, a strong positive trend from about 1920 to 1940 and another plateau from 1940 to 1980. This long-term variation indicates (within the range of the available data) a trend in low-frequency variability of precipitation. Note that this trend appears to be widespread rather than localized. Approximately 75% of the total stations in all three regions show increased variance. About 17% show no change and 8% show a decrease in variance.

The results reported here are in agreement with predictions from models operating under a climate-warming scenario. Models suggest^{6–8} that higher temperatures may result in intensification of the extremes of the hydrological cycle. Interestingly, the results reported here suggest that this increase is not synchronous with a similar increase in the global mean precipitation. Finally, I speculate on the connection between these results and those of Karl *et al.*⁴. Is their high-frequency, and my low-frequency, increase in variability part of some kind of scaling extending over a wider range in the frequency domain? If so, a formulation of such a relationship may lead to predictions of changes in variability over longer timescales for which data are not available. □

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Re–Os isotopic evidence for genesis of Archaean nickel ores from uncontaminated komatiites

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THE late Archaean greenstone terranes of Western Australia contain abundant komatiites (high-MgO lavas) hosting magmatic sulphide deposits rich in nickel, copper and platinum-group elements. Thermal erosion and assimilation of sulphidic sea-floor sediments has been proposed as a mechanism by which the komatiites were brought to sulphide saturation^{1–4}. Such models have important implications not only for the genesis of these sulphide ores, but also for interpreting the magnitude and extent of isotopic heterogeneity in the Archaean mantle. Here we report that massive, matrix and disseminated sulphide ores and a komatiite from Western Australia yield a magmatic Re–Os isochron age of $2,706 \pm 36$ Myr and a near-chondritic initial $^{187}\text{Os}/^{188}\text{Os}$ ratio of 0.10889 ± 0.00035 , whereas a proposed sulphidic sedimentary contaminant has an extremely radiogenic $^{187}\text{Os}/^{188}\text{Os}$ of 1.0983 at $2,706$ Myr. These data demonstrate that the ore-forming komatiites were derived from the upper mantle without significant contamination by radiogenic crust either before eruption or during turbulent flow at the surface. Thus, ground melting and assimilation of sulphidic sediments may not be as important in ore genesis as current theories suggest.

The 2.7-Gyr Kambalda–Perseverance–Mt Keith sulphide deposits (containing Fe, Ni, Cu and platinum-group elements) are hosted by aluminium undepleted komatiites, exhibiting chondritic $\text{Al}_2\text{O}_3/\text{TiO}_2$ ratios of 21 (ref. 5). MgO compositions of the initial komatiite lavas for both Kambalda and Perseverance vary from 28 to 32 wt% (refs 6, 7), equating to eruption temperatures of 1,560 to 1,630 °C (ref. 8). Nickel sulphide mineralization commonly occurs within trough-like embayments at the interface between the host komatiites and the underlying footwall rocks⁹. These troughs have been interpreted as primary depressions modified by thermal erosion^{2,10,11}. The formation of komatiite-hosted magmatic nickel sulphide deposits as at Kambalda, Perseverance and Mt Keith is dependent on the host magma attaining sulphide saturation. Because the sulphur content of the mantle is low (250 p.p.m.; ref. 12) and the komatiites formed at high degrees of melting, it is likely that these komatiites were sulphide-undersaturated at the time of separation from the mantle residue. Therefore, the addition of crustal sulphur via thermal erosion or ‘ground melting’ of thin, sulphidic sediments by turbulently flowing komatiite has been proposed as a mechanism by which sulphide saturation was achieved².

The Re–Os isotopic system is a powerful tracer of sulphide ore formation and can be a highly sensitive monitor of the extent of crustal involvement during ore genesis¹³. We have obtained Re–Os isotopic data for representative samples from the three largest nickel deposits in Western Australia in order to evaluate the role of crustal contamination in ore genesis and to constrain the composition of the late Archaean mantle.

Re–Os isotopic data for a representative sample of one entire komatiite flow unit (JFK1), as well as ore and sediment samples,

are given in Table 1. On a Re–Os isotopic evolution diagram, 13 Kambalda–Perseverance–Mt Keith samples containing magmatic sulphides and the unmineralized komatiite from Kambalda define an isochron with an age of $2,706 \pm 36$ Myr and a near-chondritic initial $^{187}\text{Os}/^{188}\text{Os}$ of 0.10889 ± 0.00035 , equivalent to an initial γ_{Os} value (per cent deviation in $^{187}\text{Os}/^{188}\text{Os}$ from chondritic mantle of the same age) of -0.10 ± 0.32 (Fig. 1). This age is in close agreement with U–Pb zircon ages of $2,703 \pm 4$ Myr and $2,709 \pm 6$ Myr for sediments within the komatiite pile¹⁴. The isochron demonstrates that the Re–Os isotopic systematics in these samples have remained closed for 2.7 Gyr, suggesting that greenschist to amphibolite facies metamorphism, polyphase deformation and post-magmatic gold mineralization in the Norseman–Wiluna greenstone belt did not significantly effect the Re–Os geochemistry of most of the samples analysed (see Fig. 1 inset and caption). Moreover, the measured $^{187}\text{Re}/^{188}\text{Os}$ ratios for the ores fall in a narrow range from 0.0103 to 1.72 (Fig. 1), consistent with ratios expected in ores derived from komatiitic magmas with low Re/Os ratios. This contrasts with the range in $^{187}\text{Re}/^{188}\text{Os}$ ratios displayed by komatiitic basalt and basalt-associated magmatic sulphide deposits which typically vary from 5 to 40, consistent with the higher Re/Os ratios of these magmatic systems^{15,16}.

Derivation of a komatiite by high degrees of batch melting of the Archaean mantle would result in a Re concentration of 0.4–0.7 p.p.b. and an Os concentration of 1–2 p.p.b. (compare JFK1;

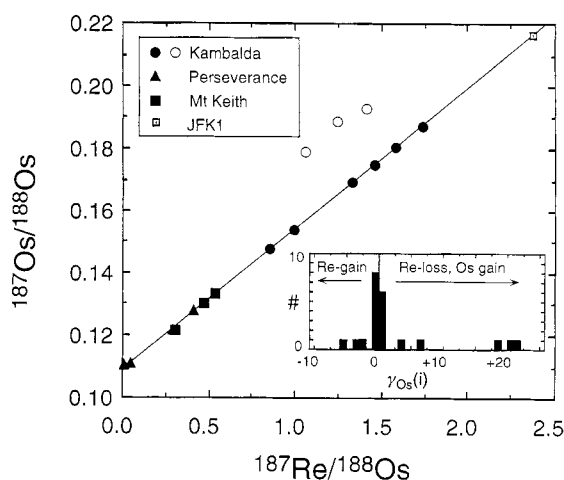


FIG. 1 Re–Os isotopic evolution diagram for 16 ore samples from Kambalda (filled circles)–Perseverance (filled triangles)–Mt Keith (filled squares; including two replicates) and komatiite JFK1 (empty square with dot). The data for 13 undisturbed ore samples and komatiite yield a model 1 isochron regression age of $2,706 \pm 36$ Myr and an initial $^{187}\text{Os}/^{188}\text{Os}$ of 0.10889 ± 0.00035 , equivalent to an initial γ_{Os} value of -0.10 ± 0.32 (see Table 1 for chondritic parameters at 2,706 Myr); mean square of weighted deviates (MSWD) is 1.12; F value = 2.22 for 20 degrees of freedom. Although the regression yields a precise initial Os isotopic composition ($\pm 0.32 \gamma_{\text{Os}}$ units), a geologically reasonable error in this parameter is $\pm 0.8 \gamma_{\text{Os}}$ units based on the difference in the calculated initial Os isotopic compositions for the two ore samples from Perseverance with the lowest $^{187}\text{Re}/^{188}\text{Os}$ ($\pm 0.8 \gamma_{\text{Os}}$ units; sample numbers Z58585 and Z58586, Table 1) as well as the difference in the calculated initial Os isotopic compositions for two complete replicate Re–Os isotopic analyses (± 0.6 – $0.7 \gamma_{\text{Os}}$ units; sample numbers Z64015 and Z00080, Table 1). Samples exhibiting apparent Re loss or gain as well as radiogenic Os gain were excluded from the regression, as illustrated by the histogram (inset) which displays the calculated initial γ_{Os} values for all mineralized samples and JFK1 at 2,706 Myr. Three samples (empty circles) lie well above the chondritic isochron and yield a model 1 ‘errorchron’ regression age of $2,472 \pm 319$ Myr, indicative of open-system behaviour. These three samples are from parts of ore systems that show significant late-stage deformation. The sulphidic sediment, with a measured $^{187}\text{Re}/^{188}\text{Os}$ ratio of 106 and a $^{187}\text{Os}/^{188}\text{Os}$ ratio of 5.91, was omitted for clarity. The calculated $^{187}\text{Os}/^{188}\text{Os}$ for the sulphidic sediment at 2,706 Myr is 1.0983, equivalent to a γ_{Os} value of +908, distinctly different from the near-chondritic initial Os isotopic composition of the ores.

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TABLE 1 Re–Os isotopic data for Kambalda–Perseverance–Mt Keith magmatic ores, komatiite and sulphidic sediment

Sample numbers	Location	Type	Re (p.p.b.)	Os (p.p.b.)	Re/Os	$^{187}\text{Re}/^{188}\text{Os}^*$	$^{187}\text{Os}/^{188}\text{Os}^\dagger$	$^{187}\text{Os}/^{188}\text{Os}(\text{i})$	$\gamma_{\text{Os}}(\text{i})^\ddagger$
Kambalda Ores									
Z64012	Victor 240/3-(U)	Massive	58.65	178.04	0.329	1.573	0.18037(126)	0.10906	+0.1
Z64012(R)	Victor 240/3-(U)	Massive		183.17			0.18888(139)		
Z64015	Victor 240/3-(U)	Massive	145.56	811.22	0.179	0.853	0.14753(103)	0.10886	–0.1
Z64015(R)	Victor 240/3-(U)	Massive	145.26	833.93	0.174	0.844	0.14771(103)	0.10943	+0.6
Z64015(R)	Victor 240/3-(U)	Massive		831.96			0.14714(103)		
Z64013	Victor 240/3-(U)	Massive	156.42	756.04	0.207	0.988	0.15410(270)	0.10933	+0.3
Z64014	Victor 240/3-(U)	Massive	62.86	180.23	0.349	1.667	0.18134(127)	0.10578	–2.9
Z64014(R)	Victor 240/3-(U)	Massive		183.85			0.18051(126)		
Z64016	Victor 240/30-(U)	Massive	86.36		0.357	1.733	0.18732(240)	0.10877	–0.2
Z64018	Victor 240/3-(U)	Matrix	39.53	165.83	0.238	1.155	0.16090(113)	0.10854	–0.4
Z58507	Victor 208/S-(U)	Massive	76.59	252.01	0.304	1.451	0.17462(122)	0.10884	–0.1
Z58542	Coronet KD8653-(D)	Massive	68.50	267.75	0.256	1.246	0.18860(132)	0.13213	+21.3
Z55765	Hunt 1201-(U)	Massive	64.92	300.18	0.216	1.055	0.17916(125)	0.13132	+20.5
Z58502	Victor 117/S-(U)	Matrix	59.53	204.50	0.291	1.416	0.19317(135)	0.12899	+18.4
Z58511	Victor 208/S-(U)	Massive	96.13	273.79	0.351	1.716	0.18102(127)	0.10326	–5.2
Z55710	Otter 2047/S(U)	Matrix	34.62	298.83	0.116	0.542	0.14056(98)	0.11599	+6.5
Z55725	Otter 2047/S(U)	Massive	272.57	1886.58	0.144	0.685	0.13798(97)	0.10694	–1.8
Mt Keith Ores									
Z64051	Mt Keith RL454	Olivine sulphide cumulate	2.12	18.81	0.113	0.533	0.13325(93)	0.10911	+0.1
Z64052	Mt Keith RL384	Olivine sulphide cumulate	1.05	16.78	0.063	0.296	0.12171(85)	0.10830	–0.6
Z00080	Mt Keith-(D)	Olivine sulphide cumulate	2.20	21.81	0.101	0.486	0.13068(91)	0.10864	–0.3
Z00080(R)	Mt Keith-(D)	Olivine sulphide cumulate	2.48	22.97	0.107	0.520	0.13271(109)	0.10913	+0.3
Perseverance Ores									
Z58581	Perseverance PU534-(D)	Olivine sulphide cumulate	2.24	25.81	0.087	0.411	0.12789(90)	0.10927	+0.3
Z58582	Perseverance PU534-(D)	Olivine sulphide cumulate	5.37	78.12	0.069	0.290	0.12142(108)	0.10828	–0.6
Z58582(R)	Perseverance PU534-(D)	Olivine sulphide cumulate		77.40			0.12136(85)		
Z58583	Perseverance PU534-(D)	Olivine sulphide cumulate	0.58	25.03	0.023	0.112	0.11711(82)	0.11205	+2.8
Z58585	Perseverance LSD224-(D)	Olivine sulphide cumulate	0.21	23.64	0.009	0.042	0.11080(78)	0.10889	–0.1
Z58586	Perseverance LSD224-(D)	Olivine sulphide cumulate	1.11	395.78	0.003	0.014	0.11030(77)	0.10969	+0.7
Silicates									
JFK1	Coronet KD8653-(D)	Komatiite	0.48	1.33	0.361	2.351	0.21607(389)	0.10953	+0.5
Z58539	Hunt KD1204-(D)	Sediment	3.48	0.27	13.13	106.1	5.9070(413)	1.09832	+908

Location-(U), underground face sample; location-(D), diamond drill core sample; (R), replicate analysis. Re–Os samples analysed by negative thermal ionization mass spectrometry (Finnigan–MAT 262 N-TIMS) following a low-blank Carius tube *aqua regia* digestion²⁸. Total procedural blanks for both Re and Os are <35 pg. Concentration data are by isotope dilution N-TIMS. Data are normalized to $^{192}\text{Os}/^{188}\text{Os} = 3.0827$, corrected for oxygen fractionation, and blank subtracted.

* Uncertainty $\leq 4\%$, including error magnification and spike calibration.

† Uncertainties for Os isotopic composition (in last significant figures in parentheses) are 2σ of the population (0.7%) for repeat DTM standard Os isotopic analyses ($n = 22$) or represent internal precision at 2 standard errors of the mean if $>7\%$.

‡ $\gamma_{\text{Os}}(\text{i})$ is defined as the deviation at the crystallization age of 2,706 Myr in parts per 100 from the isotopic composition of a 2,706-Myr chondritic reference reservoir with an initial $^{187}\text{Os}/^{188}\text{Os} = 0.1090$.

Table 1 and Fig. 2). If this magma were to attain sulphide saturation without the addition of any contaminant (for example, as a result of cooling, decompression or oxidation) it would produce an immiscible sulphide liquid with a Re and Os concentration and Re/Os ratio comparable to the Kambalda ores (Fig. 2). However, all of the Perseverance–Mt Keith ores display lower Re/Os ratios (<0.1). An important variable in controlling the Re and Os composition of an immiscible sulphide liquid is the mass ratio of silicate liquid to sulphide liquid or *R*-factor¹⁷. Assuming silicate melt/sulphide melt partition coefficients for Re and Os within the same order of magnitude, then an increase in *R*-factor

to 5,000 only marginally reduces the Re/Os ratio (Fig. 2). In this case, the low Re/Os ratios for Perseverance–Mt Keith ores cannot be attributed to *R*-factor variation alone. However, if silicate melt/sulphide melt partition coefficients for Re and Os are disparate by several orders of magnitude, then the range in Re/Os may be explained by *R*-factor variation (Fig. 2). Alternatively, another process such as fractional crystallisation of monosulphide solid solution from the immiscible sulphide liquid could produce the distribution of data towards low Re/Os. Experimental data suggest that Os is compatible in monosulphide solid solution¹⁸. If Re is incompatible or less compatible than Os then sulphide

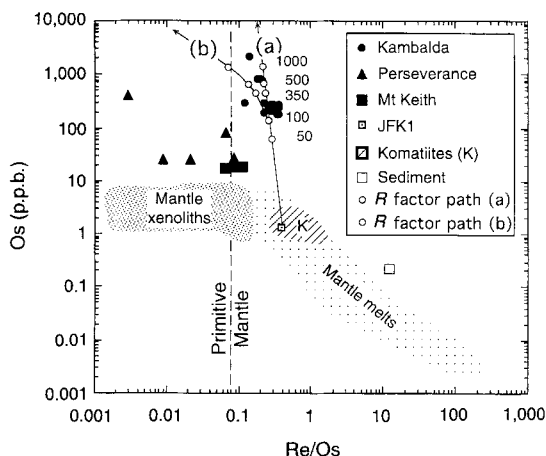


FIG. 2 Plot of Os (p.p.b.) against Re/Os ratio, showing Re–Os evolution as a function of komatiite melt extraction from the mantle and subsequent immiscible sulphide liquid formation. Data for Kambalda (filled circles)—Perseverance (filled triangles)—Mt Keith (filled squares) ore samples, komatiite JFK1 (empty square with dot) and sulphidic sediment (empty square) shown for comparison. Core segregation at 4.56 Gyr (ref. 29) followed by addition of chondritic material³⁰ to the Earth's surface leads to the formation of primitive mantle¹² (Re, 0.28 p.p.b.; Os, 3.4 p.p.b.; Re/Os, 0.08). Early crust formation results in 1–10% depletion of primitive mantle³¹ to form depleted mantle (Re, 0.24 p.p.b.; Os, 3.45 p.p.b.; Re/Os, 0.07). Aluminium-undepleted komatiites (hatched field 'K') form at 2.7 Gyr by high degrees of batch melting of depleted mantle (Re, 0.43 p.p.b.; Os, 1.15 p.p.b.; Re/Os, 0.4) leaving a harzburgite residue with chemistry similar to 'mantle xenoliths' (Re, 0.04 p.p.b.; Os, 5.76 p.p.b.; Re/Os, 0.007). Modelled *R*-factor evolution pathways for an immiscible sulphide liquid derived from a komatiite with the composition of JFK1 are shown. Model (a) assumes that the sulphide liquid/silicate liquid partition coefficients (*D*) for both Re and Os are the same order of magnitude, where $D \approx 10^5$. Model (b) assumes that partition coefficients for Re and Os are disparate by several orders of magnitude, where $D_{\text{Re}} \approx 10^2$ and $D_{\text{Os}} \approx 10^5$. Fields for mantle xenoliths and mantle melts, komatiites and basalts are shown for reference^{32–34}.

'cumulates' would have low Re/Os ratios, as is observed in the Perseverance–Mt Keith nickel ores (Fig. 2).

A finely laminated pale grey siliceous sulphidic sediment from the interface between the host komatiites and underlying basalts is not isochronous with the ores (Fig. 3). This contact sediment is typical of the proposed contaminants at Kambalda², with a mineralogy dominated by quartz and albite, subsidiary bedding-parallel hydrothermal pyrrhotite and pyrite and a sulphur content of 4.5 wt%, close to the average value for Kambalda contact sediments (4 wt%)¹⁹. This sediment exhibits a very high Re/Os ratio of 13 (Fig. 2), has an extremely radiogenic $^{187}\text{Os}/^{188}\text{Os}$ of 1.0983 at 2.7 Gyr (equivalent to a γ_{Os} value of +908; Table 1) and a chondritic mantle model age of 3.2 Gyr. The presence of an old and very radiogenic component in the Os of this sediment is consistent with the radiogenic initial Pb isotopic composition of sediments from Kambalda²⁰ as well as the identification of inherited zircons with ages up to 3.45 Gyr in the volcanic pile²¹. The new Re–Os isotopic data for the sediment is further evidence for

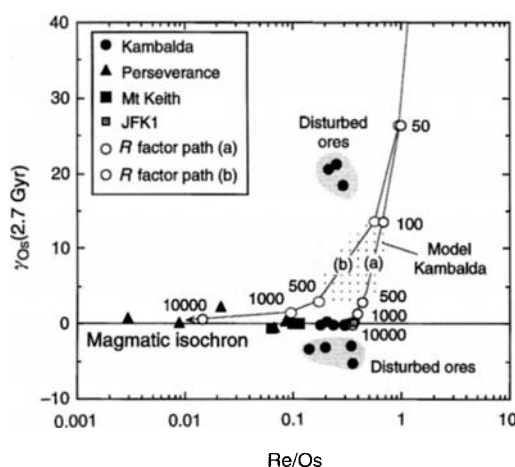


FIG. 3 Plot of calculated initial γ_{Os} against Re/Os ratio for Kambalda (filled circles)—Perseverance (filled triangles)—Mt Keith (filled squares) ore samples and komatiite JFK1 (empty box with dot). The horizontal line represents the isochron initial of $\gamma_{\text{Os}} = -0.10$. The dynamic ore-forming process has been modelled in Re–Os isotope space as a function of the mass ratio of komatiite liquid to immiscible sulphide liquid (R -factor), using the following equation:

$$\left(\frac{^{187}\text{Os}}{^{188}\text{Os}}\right)_{\text{tot}} = \left[\frac{((C_m D_n (R+1)/(R+D_n))}{((C_m D_n (R+1)/(R+D_n)) + C_{sm})} \right] \times \left[\left(\frac{^{187}\text{Os}}{^{188}\text{Os}}\right)_{\text{pm}} - \left(\frac{^{187}\text{Re}}{^{188}\text{Os}}\right) (e^{\lambda t} - 1)_{\text{pm}} \right] + \left[1 - \frac{((C_m D_n (R+1)/(R+D_n))}{((C_m D_n (R+1)/(R+D_n)) + C_{sm})} \right] \times \left[\left(\frac{^{187}\text{Os}}{^{188}\text{Os}}\right)_{\text{psm}} - \left(\frac{^{187}\text{Re}}{^{188}\text{Os}}\right) (e^{\lambda t} - 1)_{\text{psm}} \right]$$

where C_m indicates the common Os concentration in source magma, C_{sm} the common Os concentration in sediment sulphide component, D_n the sulphide liquid/silicate liquid partition coefficient for osmium, pm the measured isotopic composition of the source magma, psm the measured isotopic composition of sulphidic sediment, λ the Re decay constant of $1.64 \times 10^{-11} \text{ yr}^{-1}$ (ref. 35), t the closure age of the isotope system and tot is the initial isotopic composition of immiscible sulphide liquid for any given R -factor. Partition coefficients for model (a) and (b) are the same as for Fig. 2. Open circles on model curves represent the isotopic composition of the immiscible sulphide liquid, and the adjacent numbers represent the R -factor. The predicted range in R for Kambalda is $500 > R > 100$ (ref. 36) and is shown stippled as 'Model Kambalda'. 'Disturbed ores' are either those samples which appear to have lost Re and/or gained radiogenic Os and thus have anomalously high calculated initial Os isotopic compositions, or those samples which have gained Re and thus have anomalously low calculated initial Os isotopic compositions (see Fig. 1 inset).

eruption and emplacement of the volcanic succession in an ensialic setting²².

Simple two-component mixing of a komatiite, with a chondritic Os isotopic composition, and a sulphidic sediment, with the geochemical and isotopic characteristics outlined above, would have resulted in the formation of highly radiogenic ores. Using model crustal contamination parameters²³, the ores should have initial γ_{Os} values of +6 to +35. Clearly, this process is not supported by the Re–Os isotopic data for these ores. A more realistic volcanological process is conceivable whereby assimilation of sediment occurs followed by immediate segregation of the sulphide liquid component from the silicate component, forming a basal sulphide layer²⁴. The ore elements would have partitioned from the flowing komatiite into the sulphide layer as a function of lava 'flow-through' and thus R -factor. Using an R -factor of 250, modelling of this dynamic volcanological process predicts that the ores should have initial γ_{Os} values of $>+5$ (Fig. 3). Of equal importance for this study, ores generated by 'ground melting' and assimilation of sulphidic sediments would have high $^{187}\text{Re}/^{188}\text{Os}$ ratios due to the high Re concentrations in the sediments relative to the komatiite (Table 1 and Fig. 3). Thus, even 2.7-Gyr sulphidic sediments with chondritic initial γ_{Os} values would impart significant crustal Re to the ores, which is not observed.

The Re–Os isotopic data presented here thus preclude significant bulk assimilation of sulphidic sediments of the Kambalda-type analysed in this study and may rule out contamination of the komatiites even by sediments that had near-chondritic γ_{Os} values. This is consistent with the result of mass-balance calculations²⁵ which show that there is insufficient sulphide missing from the local site of ore deposition to generate the amount of magmatic sulphides observed at Kambalda. However, the generation of magmatic sulphide ores by crustal contamination cannot be ruled out entirely. This study demonstrates that the potential contaminant must have had a non-radiogenic Os isotopic composition at 2.7 Gyr, a low Re/Os ratio, and low concentrations of both Re and Os. None of these characteristics match the proposed sedimentary contaminant or any modern sedimentary analogue²⁶. Furthermore, for crustal contamination to be consistent with our data, the Re sulphide melt/silicate melt partition coefficient must also be significantly lower than that for Os and outside the range predicted by magmatic sulphide ores worldwide. Thus, the Re–Os geochemistry of these ores is entirely consistent with derivation from uncontaminated komatiites. The Re–Os isotopic data coupled with the volcanological modelling indicate that the composition and economic concentration of an immiscible sulphide liquid may owe more to processes involved in transport and deposition of this liquid than the timing and mechanism of sulphide saturation. Ultimately, sulphide saturation in the Kambalda, Perseverance and Mt Keith ore systems may have been achieved via changes in intensive parameters of the lava (cooling or decompression) or changes in compositional parameters transparent to the Re–Os isotopic system ($f_{\text{O}_2}/f_{\text{S}_2}/f_{\text{H}_2\text{O}}$). Our Re–Os isochron for Kambalda, Perseverance and Mt Keith yields a precise near-chondritic initial Os isotopic composition and, by analogy with modern Os mantle reservoirs²⁷, is consistent with the derivation of these ore-forming komatiites from a depleted upper-mantle source. □

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Bite-force estimation for *Tyrannosaurus rex* from tooth-marked bones

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WHETHER tyrannosaurs occupied predatory or scavenging niches has been debated for nearly a century^{1–5}. Palaeontologists have turned to the study of dental morphology to address this question, but the results have been highly disparate. Some contend that the tyrannosaur dentition was very strong and well suited for engaging and killing herbivorous dinosaurs^{6,7}. Others posit that tyrannosaurs ate carrion, because their teeth and/or jaws would fail during struggles with prey^{2,3}. The discovery of skeletal remains with bite marks from *Tyrannosaurus rex*⁸ makes it possible to estimate, through indentation simulations on bovine ilia, the bite forces produced by *T. rex* during feeding. The estimates (6,410 to 13,400 N) rival the largest bite forces determined for any taxon to date and suggest that *T. rex* had very strong, impact-resistant teeth. Although these data do not prove that *T. rex* was predominantly predacious, they indicate that its dentition could probably withstand the stresses associated with prey capture.

A recently unearthed *Triceratops* sp. pelvis from the Hell Creek Formation of Montana (USA) bears dozens of large bite marks (Fig. 1)⁸. Casts of some of the deeper punctures show that an adult *T. rex* produced the marks using its longer anterior caniniform

teeth⁸. The bitten bones are predominantly composed of cancellous bone tissue, capped only by a thin layer of dense cortical bone⁸. On the basis of these marks, it is difficult to gauge whether the teeth that produced the bite marks were particularly robust. We attempted to quantify the forces that the tyrannosaur dentition absorbed when biting the *Triceratops* ilium, by using laboratory simulations. We contrasted the results with those for extant taxa to place them in a comparative context, and assessed the functional and behavioural implications of these comparisons.

Using histological examination we determined that extant bovine ilia exhibit comparable microstructure to *Triceratops* ilia. Consequently, bovine ilia were used to model the bitten *Triceratops* bones. Sections of ilia with cortices of varying thickness were penetrated with a *T. rex* tooth replica to a depth of 11.5 mm (the depth of the deepest ilium bite mark⁸) using a servohydraulic mechanical loading frame. The forces produced throughout these simulations were recorded. When indented, the bovine ilia exhibited localized crushing as the only mode of failure, and the punctures produced were comparable in morphology to the *T. rex* bite marks. The forces during testing increased with increasing penetration depths (Fig. 2). Peak forces ranged from 1,900 to 15,100 N (Fig. 3). A positive correlation between peak penetration force and cortical thickness was found (Fig. 3).

A bone sample removed from the bitten *Triceratops* ilium within 2 cm of the deepest bite mark (11.5 mm) revealed a cortical thickness of 2.5 mm. From a linear regression of our data (Fig. 3), we determined that roughly 6,410 N of force was required to produce the bite mark. Estimates as great as 13,400 N for posterior teeth were obtained when biting velocity, energy absorption by flesh, and the mechanical advantage of posterior teeth relative to more anterior teeth were taken into consideration (Fig. 3).

These bite-force estimates make it possible to evaluate speculations on tyrannosaur tooth strength and potential behaviours using comparisons with extant taxa. The largest maximum bite force measurements or estimates for extant vertebrates at posterior tooth positions are: 550 N for Labrador dogs⁹, 749 N for humans¹⁰, 1,412 N for wolves¹¹, 1,446 N for dusky sharks (location of force measurement within jaw not given)¹², 1,712 N for orangutans¹³, 4,168 N for lions¹¹, and 13,300 N for American alligators¹⁴ (K. A. Vliet, personal communication). Using bite force as a relative indicator of dental strength, the results suggest that *T. rex* teeth were as strong as, or in most cases substantially stronger than, those of any extant taxa tested to date. Consequently,

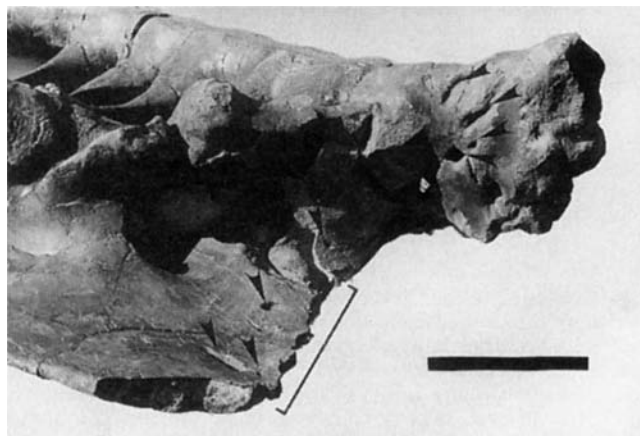


FIG. 1 *Triceratops* sp. pelvis in ventrolateral view bearing bite marks from an adult *Tyrannosaurus rex*. The sacrum and left ilium (Museum of the Rockies specimen MOR 799, Montana State University, Bozeman, MT) have 58 definitive bite marks attributable to 'puncture and pull' biting behaviour by the feeding tyrannosaur(s)⁸. Arrows denote some of the more conspicuous bite marks. Brackets bound a region where the tyrannosaur(s) removed approximately one-sixth of the anterior portion of the ilium by means of repetitive biting. Scale bar, 25 cm.

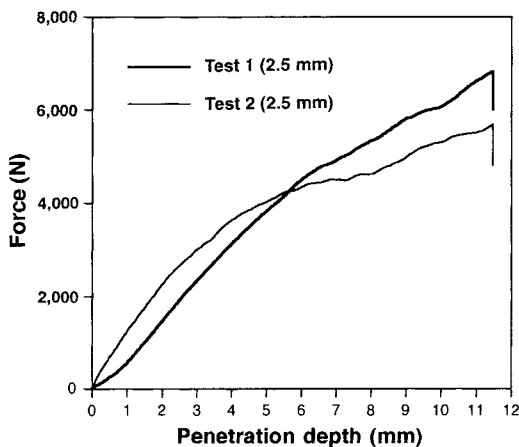


FIG. 2 Typical force against penetration curves produced during the penetration of bovine ilia by an adult *Tyrannosaurus rex* tooth replica. The two curves represent simulations conducted on samples with 2.5-mm thick cortices.

METHODS. Bovine ilia were used in the simulations because their histological structure (a fibrolamellar cortex overlying cancellous bone²⁶) was found to match that of the *Triceratops* ilium. Bone sections $10 \times 50 \times \sim 3.0$ cm with cortices ranging from 0.5 to 5.5 mm in depth (the range of initial cortical-thickness estimates based on gross morphology) were mounted on a servohydraulic mechanical loading frame (MTS Bionix, Minneapolis) and penetrated with an aluminium-bronze *T. rex* tooth replica. The replica was cast from an actual adult *T. rex* maxillary tooth, after casts made from some of the deeper bite marks revealed the size and shape of the teeth that had impacted the pelvis⁸. The replica was penetrated into the ilia sections at 1 mm s^{-1} to a depth of 11.5 mm, equivalent to the maximum depth of the deepest ilium bite mark⁸. Forces were measured with an MTS 25 N strain-gauge-based axial load cell accurate to 0.2%. The forces increased with increasing penetration depth even after the cortical layer had been perforated and the underlying cancellous bone was being crushed. The increase in force with penetration depth is attributed to a greater cortical surface area coming into contact with the semi-conical penetrator tooth as it descended through the ilia.

speculations that their dentition was mechanically weak were not supported.

Peak bite-force estimates for large American alligators (*Alligator mississippiensis*) are within the range we calculated for a feeding *T. rex*. This taxon shares many dental attributes with *T. rex* (including thecodont implantation^{15,16}, stout semi-sharp caniniform teeth that are transversely rounded^{6,7,16}, and nearly identical histological structures^{17,18}). These morphological similarities imply similarity in function¹⁹. Alligators use their teeth to procure large prey and to engage conspecifics during confrontations²⁰. Both activities demand teeth that can sustain large compression and bending forces, particularly because impacts with bones are frequent²⁰. The bite-force estimates and tooth mark evidence show that *T. rex* teeth could similarly withstand large bite forces and sustain repetitive bone impacts. Therefore, it is not unreasonable to suspect that the *T. rex* dentition could be used in behaviours similar to those of alligators, and with some mechanical safety²¹. Physical evidence supports this reasoning. Bony calli on adult tyrannosaur crania attest to biting injuries during intraspecific aggression^{22,23}, and a healed hadrosaur tail injury has been attributed to biting by a *T. rex* during a failed predation attempt⁵.

Although our data suggest that *T. rex* could produce enormous bite forces and possessed a dentition that could endure stresses associated with prey struggles, they by no means prove that *T. rex* was predacious. Indeed it could be argued that these characteristics enhanced their utilization of scavenged carcasses. Nevertheless, these results refute assertions that *T. rex* was mechanically limited by its dentition to scavenging carrion. We contend that if *T. rex* could consistently manoeuvre into a position to engage prey with its dentition, it could have exploited a predatory niche.

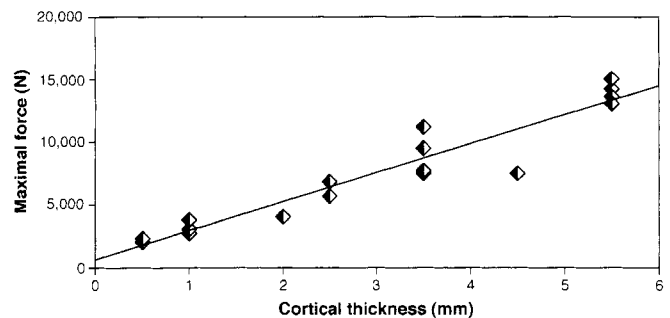


FIG. 3 Maximum penetration force values for an adult *Tyrannosaurus rex* tooth replica impacted through bovine ilia with varying cortical thickness. Peak penetration forces increased with increasing cortical thickness ($y = 2305.402x + 646.634$, $r^2 = 0.91$).

METHODS Because of the 7-fold range of peak force values in the simulations, it was necessary to obtain precise measurements of the cortical depths penetrated by the *T. rex* teeth. A bone sample taken adjacent to the deepest bite mark revealed a 2.5 mm cortical thickness. From the regression equation, approximately 6,410 N of force was required to produce the bite mark. This bite mark was made by one of the tyrannosaur's longer caniniform teeth⁸, probably a tooth between the fourth and seventh maxillary positions (based upon American Museum of Natural History specimen AMNH 5027, New York). To account for the relative mechanical advantage of more posteriorly positioned teeth²⁷, moment calculations were used to calculate the simultaneous forces produced at the most posterior tooth positions. Values ranging from 7,870–10,300 N were projected, assuming 6,410 N of force were produced simultaneously by teeth from the fourth to seventh tooth positions. Because bone strength increases with strain rate²⁸ and the penetration rate of the tooth replica was just 1 mm s^{-1} , it is likely that the simulation force values underestimated actual forces. A tooth-impact velocity of 10 mm s^{-1} for a biting tyrannosaur (based on extant large reptile feeding; G.M.E., personal observations) would have required $\sim 20\%$ more bite force²⁸. Adhering flesh⁸ may have absorbed another 10% (or more) of the initial bite force²⁹. These considerations suggest that bite forces as high as 13,400 N could have been produced by an adult *T. rex* during feeding. Greater forces may have been possible during snapping bites or those involving bodily inertia to augment tooth penetration. Such biting is characteristically used when prey are seized initially (G.M.E., personal observations of reptilian feeding). Taphonomic interpretations suggest that the bite marks on the *Triceratops* ilium were not the result of this behaviour⁸. Additionally, if the tyrannosaur's contralateral teeth were used when the deepest bite mark was made, greater bite forces may have been generated than those we estimated^{13,30}. This is indeterminable from MOR 799.

It has been shown recently that theropod bite marks are much more common in the fossil record than was once suspected^{18,24,25}. Consequently, the methods used here could be used to assess bite-force estimates for other tyrannosaur individuals, as well as for many theropod species. Such data would greatly augment our understanding of dinosaur tooth form and function, the physical capacities of their teeth and jaws (ontogenetically and interspecifically), and provide new insight into the musculoskeletal biomechanics of dinosaur crania. □

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Tracking the evolution of warning signals

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EVOLUTIONARY studies are hampered by a lack of experimental ways in which to test past events such as the origination of aposematism^{1–7}, whereby unpalatable or poisonous prey signal their unprofitability, often by being warningly coloured. Inexperienced predators do learn to avoid unpalatable prey as a result of such signals^{8–10}, but in addition there may be an inherited cautiousness about attacking when common or conspicuous warning signals are evident^{11–16}. As current predators are not naive in the evolutionary sense, it is still not resolved^{3–7,17,18} whether aposematism originated only in aggregations of prey^{19,20} or among solitary prey as well^{21–23}. Here we explore this controversy in evolutionarily naive predators by creating a novel world with warning signals not found in the environment. Initially, the aggregation of prey favoured the warning signals supporting Fisher's view²⁴ of kin aggregations as the evolutionary starting point of aposematism. However, once predators had experienced warning signals, pre-existing avoidance seemed to facilitate evolution of Müllerian mimicry complexes²⁵ with similar types of signals even among solitary prey.

On the basis of the fact that hand-reared predators with no experience of aposematic prey are not naive in the evolutionary sense, we created a world in which there were two types of symbols (crosses and filled squares) that were switched to form the background or the warning signal. As the symbols were not present during the evolutionary history of the predators, we assume that the experiments illustrate the initial conditions experienced by the first aposematic prey individuals. We started from the situation in which an unpalatable prey evolves a warning signal, then continued to test conditions for the evolution of similar signals in other unpalatable prey items (Müllerian mimicry)²⁵. We used great tits (*Parus major*) caught in the wild as predators. To enhance the image of a novel world, all prey items were artificial. As initial prey we used items made of a piece of rye straw (a hollow stem of dead cultivated rye) filled with animal fat. White paper 'wings' at each end of the straw piece had either a cryptic signal similar to the background or a warning signal different from the

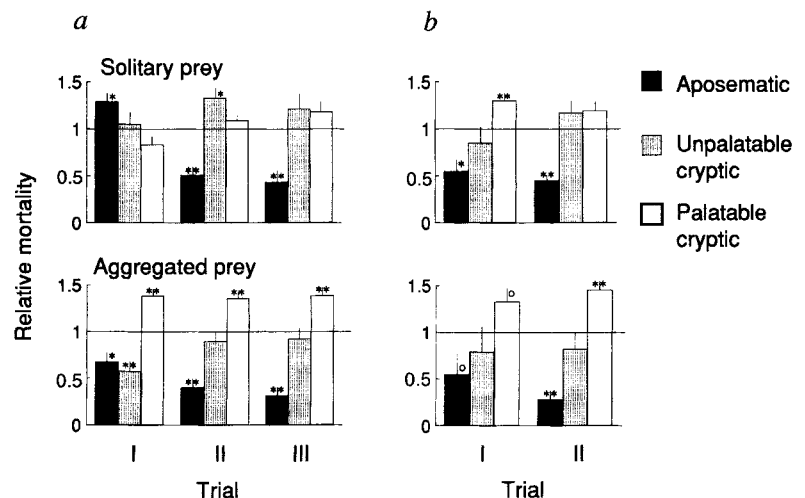
background. To make the items unpalatable we added chloroquine to the fat. As secondary prey, we used pieces of almond with the symbol glued on the slice.

In the 'initial origin' experiment, the prey were either dispersed as single items or aggregated with four similar items clumped close together (Fig. 1). As the idea was to determine how the warning signal initially enhances the survival probability of prey that have just acquired unpalatability^{19,20}, we had three types of randomly distributed prey: palatable controls with the cryptic symbol (16 items), unpalatable prey with the cryptic symbol (8 items), and unpalatable prey with the symbol differing from that of the background as the aposematic prey (8 items). Each individual tit had been trained to eat straw items with the cryptic symbol, and they were allowed to search for one hour in the test room. The procedure was repeated on three consecutive days (trials I–III). Conspicuousness or the type of signal itself did not influence the innate preferences between the two types of signals. Another set of eight tits were presented with two prey items (only 2 cm apart) and the same choice situation was repeated four times. The birds did not prefer any of the symbols (first choices for squares, 15, and crosses, 17; binomial test, $P = 0.86$) or the symbol conspicuous-

		Type of dispersion	
		Solitary prey	Aggregated prey
Background	Cross	 palatable 16x1	 palatable 4x4
		 unpalatable 8x1	 unpalatable 2x4
		 aposematic 8x1	 aposematic 2x4
	Square	 palatable 16x1	 palatable 4x4
		 unpalatable 8x1	 unpalatable 2x4
		 aposematic 8x1	 aposematic 2x4

FIG. 1 The experimental set-up to test the origin of warning signals among straw prey. Each individual great tit was randomly assigned to one of the four treatments shown. The background was either a cross or a square and the prey were presented either solitary or in aggregation. In each case, there were 16 palatable cryptic prey, 8 unpalatable cryptic prey and 8 unpalatable signalling prey (aposematic). Palatable items consisted of animal fat in a 6-mm rye straw with paper wings. Unpalatable items had a 12% concentration of chloroquine. Prey items were randomly placed on a 2 × 2 m floor of an aviary, where there were 6 × 6 = 36 pieces of white A4 paper (21 × 30 cm) with symbols as the background. To train the tits to use their cryptic items, they were offered two palatable pieces of straw filled with fat on two consecutive days. Before the trials, the tits were not allowed to feed for two hours, and each trial lasted one hour. We used only the 12 first items in the analyses, but in the first trials only the 6 first items were included to obtain the initial predation risks before birds could learn the signals. In the second experiment, almond slices were reduced to ~ 6 × 6 mm, with symbols glued to each slice with non-toxic glue. Unpalatable slices were dipped in a 40% solution of chloroquine.

FIG. 2 Relative mortality for aposematic, unpalatable cryptic and palatable cryptic prey items when presented solitarily dispersed or in clumped aggregations. The proportions of killed items in each group were divided by the randomly expected proportions, which were 0.25 for aposematic and unpalatable cryptic items, and 0.50 for palatable cryptic items. In this way, the average mortality over the three types of items is standardized to unity. In the first stage (a) with artificial straw prey, the experiment was repeated three times with the same birds, and in the second stage (b), the same predator individuals each encountered a similar set-up of slices of almond with similar signals in two consecutive trials. Standard errors are shown; asterisks indicate two-tailed significance (circle symbol, $P < 0.10$; * $P < 0.05$; ** $P < 0.01$) of a difference from the average mortality of all items, scaled to unity as indicated by the horizontal lines. We used repeated measures ANOVA for arcsin-square-root transformed proportions of each type of item to be 'killed'. The type of background (square or cross) had no effect in either setup for any of the three prey types ($P > 0.50$), so for simplicity we have combined the presentation of data. In the 'initial origin' experiment (a), for aposematic items both the type of prey dispersion ($F_{(1,23)} = 8.47$, $P < 0.01$) and the trial number ($F_{(2,46)} = 12.10$, $P < 0.01$) indicated significant effects. Likewise, type of prey dispersion ($F_{(1,23)} = 15.86$, $P < 0.01$) and trial number ($F_{(2,46)} = 5.32$, $P < 0.01$) had a significant effect on the proportion of unpalatable cryptic items being killed. For the cryptic palatable items, the type of dispersion was significant ($F_{(1,23)} = 24.38$, $P < 0.001$) but trial



number was not ($F_{(2,46)} = 2.39$, $P < 0.10$). In the 'secondary origin' experiment (b) the proportion of aposematic items handled was not significantly dependent on the type of prey dispersion ($F_{(1,17)} = 1.85$, $P = 0.19$, repeated measures ANOVA) or the trial number ($F_{(1,17)} = 0.37$, $P = 0.58$).

ness (similar to background, 16; different from background, 16; binomial test, $P = 1.00$).

In the 'initial origin' experiment, if the prey were aggregated, tits left the unpalatable groups after tasting the first prey items (Fig. 2a, trial I). Thus, the selective advantage of aggregation appeared initially for both the aposematic prey and the unpalatable cryptic prey. In later trials (II–III), whereas unpalatable cryptic items retained their advantage over palatable cryptics, aposematic items were used much less as a consequence of birds learning to avoid the signalling prey. If the prey were solitary, there was initially a disadvantage for warning signals (trial I), but selective advantage appeared in later trials after predators had previous experience with similar phenotypes. Altogether, this suggests that initially aggregation would have been beneficial for the aposematic prey, seemingly supporting Fisher's view of kin selection. Indeed, unpalatability alone would have selected for gregariousness which subsequently facilitates the evolution of warning signals. Although our experiment assured that the signals were novel, the predators could have experienced aggregated unpalatable prey in the wild, and aggregation alone might act as a cue of unpalatability^{2,4,26}. However, we tested aggregated unpalatable prey together with aggregated palatable prey, so tits never had a choice between aggregated and solitary items. Furthermore, among the first attacks only, the items with warning signals were equally often attacked first in both types of prey dispersions (in 9 out of 14 cases, an aposematic item was attacked before any of the unpalatable cryptic items among solitary prey, and in 10 out of 14 cases among aggregated prey).

In the second series of experiments, the same birds were feeding on similarly arranged artificial-prey items of sliced almonds. Thus, this prey had a totally different appearance, but the cryptic and aposematic symbols were exactly the same as in the initial prey. Within the first trial, the risk of being 'killed' was highly reduced among the dispersed prey items (Fig. 2b). Predators had avoided the signalling, unpalatable items in the first encounters, suggesting that, in Müllerian mimicry, aggregation of the prey is not essential. Thus, the controversy^{2–7,14–22,26,27} over the role of prey aggregation may well originate from different conditions in previous experiments. If the situation was unusual, then the necessity of aggregation may have been important, but if the experiments used traditional signals, the benefit could have appeared even among the solitary prey items. In our experiment, the signal was exactly

the same but the prey items were very different in appearance. Thus, Müllerian mimicry may also operate effectively for imperfect mimics, which indeed may have been the scenario in which many of the presently occurring aposematic forms evolved.

Our experiments suggest a possible route for the initial evolution of aposematism in the world with evolutionarily naive predators. Unpalatability alone selected for gregariousness, as predators would just leave the aggregations after tasting the first items. Once the prey were aggregated, selection would have instantly favoured the appearance of a warning signal to allow the predators to learn to associate the signal with unpalatability. The assumption that aggregation and kin grouping would have been closely associated makes sense, because an aggregation may arise from the habit of a single female to lay her clutch in one place²⁸. However, it is not obvious that we need to invoke kin selection to explain the evolution of warning signals^{4,18}. It is the aggregation itself that helps the already unpalatable aposematic prey types. Finally, once predator populations have experienced the world with aposematic prey, aggregations may not be necessary for successful evolution of common warning signals in a new species. □

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The PIE-1 protein and germline specification in *C. elegans* embryos

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TOTIPOTENT germline blastomeres in *Caenorhabditis elegans* contain, but do not respond to, factors that promote somatic differentiation in other embryonic cells^{1,2}. Mutations in the maternal gene *pie-1* result in the germline blastomeres adopting somatic cell fates³. Here we show that *pie-1* encodes a nuclear protein, PIE-1, that is localized to the germline blastomeres throughout early development. During division of each germline blastomere, PIE-1 initially associates with both centrosomes of the mitotic spindle. However, PIE-1 rapidly disappears from the centrosome destined for the somatic daughter, and persists in the centrosome of the daughter that becomes the next germline blastomere. The PIE-1 protein contains potential zinc-finger motifs also found in the mammalian growth-factor response protein TIS-11/NUP475 (refs 4–7). The localization and genetic properties of *pie-1* provide an example of a repressor-based mechanism for preserving pluripotency within a stem cell lineage.

In the development of all animal embryos, certain cells must remain totipotent to form the reproductive cells, or germ cells, for the next generation. The germ cells of the nematode *C. elegans* arise from a sequence of unequal divisions during early embryogenesis (Fig. 1). After each of these divisions, one daughter will produce only somatic cell types, such as muscle cells or neurons; this daughter can be described as a somatic blastomere. The other daughter will produce germ cells in addition to somatic cells, and thus can be described as a germline blastomere.

Studies on cell-fate determination in *C. elegans* embryos have shown that some maternally expressed factors that function in somatic development also are present in the germline blastomeres^{1,2}. For example, development of the somatic blastomere in a four-cell embryo, EMS, but not the sister germline blastomere P₂ requires the maternally expressed transcription factors SKN-1 (refs 9, 10) and POP-1 (ref. 2), although these proteins are present in P₂ at the same levels as in EMS^{1,2}. P₂ seems to be prevented from responding to these factors by *pie-1*(+) activity^{2,3}. The *pie-1* gene is

expressed maternally; in *pie-1* mutant embryos, the P₂ blastomere does not produce germ cells, and instead undergoes a pattern of somatic differentiation similar to a wild-type EMS blastomere³. In *pie-1*; *skn-1* double mutants, the germline blastomere in eight-cell stage embryo, P₃, does not produce germ cells and instead undergoes somatic differentiation similar to a wild-type somatic C blastomere³. These results suggest that *pie-1*(+) activity in wild-type development prevents P₂ and P₃ from responding to factors that determine the EMS and C fates, respectively.

In initial experiments to clone the *pie-1* gene, we found that the genetic position of *pie-1* coincided with a region of the physical map of the *C. elegans* genome for which there were no available genomic clones or sequences. We therefore screened for rare, spontaneous *pie-1* alleles in a strain with a high frequency of transposon mutagenesis. A single, transposon-induced allele, *pie-1*(*zu177::Tc1*), was obtained from a screen of ~500,000 animals¹¹, and was used to clone the *pie-1* gene (Fig. 2). A full-length *pie-1* complementary DNA sequence was used to search for related products in the protein and nucleic acids databases (Fig. 2). The *pie-1* gene encodes a novel protein, but has two copies of a motif originally described in the TIS-11/NUP475 family of proteins^{4–7} (Fig. 2). This motif consists of a pattern of three cysteine and one histidine residues (Fig. 2b) and has been proposed to form a zinc-binding domain, or finger⁴. This motif may have an ancient origin; similar sequences are found in many animal, plant and fungal proteins, and are present in the predicted products of at least 10 genes identified by the *C. elegans* genome sequencing project (C.C.M., unpublished observations). Examples include the mammalian protein U2AF35 (ref. 12) and the *Drosophila* proteins Suppressor of Sable¹³ and Unkempt¹⁴. Although U2AF35 and Suppressor of Sable have been implicated in pre-messenger RNA splicing, a biochemical function has not been established for this motif.

The *pie-1* mRNA is expressed maternally and is detected in gonads, oocytes and in all blastomeres until the four-cell stage of embryogenesis; during later stages the *pie-1* mRNA is degraded in somatic blastomeres but retained in the germline blastomeres (G. Seydoux, personal communication). This distribution has been reported previously for several other unrelated mRNAs in the early *C. elegans* embryo, and may represent the general pattern of degradation for non-localized maternal mRNAs¹⁵.

To examine the distribution of the PIE-1 protein we raised antibodies against three different PIE-1-specific peptides (Fig. 3). Sera against all three peptides reveal a similar embryonic and mitotic distribution of PIE-1 protein. PIE-1 protein first becomes detectable at low levels in the posterior cytoplasm of one-cell stage embryos (data not shown). In two-cell stage embryos, PIE-1 is

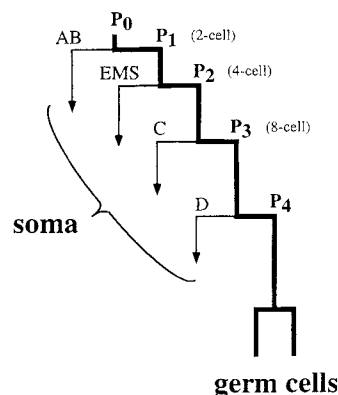


FIG. 1 The origin of germ cells in *C. elegans*, beginning with the fertilized egg (P₀). Each of the early divisions, represented by horizontal bars, produces a somatic blastomere (P₁, P₂, P₃ or P₄). Subsequent divisions of the somatic blastomeres are not shown.

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present in the nucleus and cytoplasm of the germline blastomere P_1 , but at low or non-detectable levels in the somatic blastomere AB (Fig. 3a). In four-cell stage embryos, PIE-1 is present in P_2 , but at low or non-detectable levels in its somatic sister EMS (Fig. 3b). As described previously¹, the SKN-1 protein is present in P_1 , but when P_1 divides SKN-1 is present at equal levels in both of the P_1 daughters, EMS and P_2 . Thus the asymmetrical localization of PIE-1 to P_2 nucleus provides a simple explanation of the observation that *pie-1(+1)* activity seems to prevent the activity of the SKN-1 transcription factor in P_2 , but not in EMS³. PIE-1 protein remains localized to the germline blastomeres at each of the subsequent cleavages: In 8-cell and 16-cell stage embryos PIE-1 is detected only in P_3 and P_4 , respectively (Fig. 3c, d). When P_4 divides, both of its daughters are germ-cell precursors, and PIE-1 is detected in the nuclei of both daughters (Fig. 3e).

PIE-1 appears to be associated with punctate structures in the cytoplasm of germline blastomeres (Fig. 3). Double-labelling experiments indicate that these structures are P granules¹⁶, which are germline-associated particles of unknown function and composition (data not shown). P granules are present at all stages of the *C. elegans* life cycle in either mitotic germ cells (larvae), mature germ cells (oocytes in adults), or in germline blastomeres (in embryos)¹⁶. In contrast, PIE-1 is found in P granules only in germline blastomeres, beginning with the P_1 blastomere in the two-cell stage embryo.

Because the *pie-1* mRNA is present in all blastomeres in two-cell- and four-cell-stage embryos, translational or post-translational mechanisms must control the asymmetrical distribution of PIE-1 protein during these stages. For example, the association of PIE-1 with P granules during the early cell divisions could serve

to localize PIE-1 to the germline blastomeres. A second possible contribution to PIE-1 asymmetry is suggested by the finding that PIE-1 antisera specifically stain the centrosomes of dividing germline blastomeres (Fig. 4). We have examined the intracellular distribution of PIE-1 as each germline blastomere divides, and find a common sequence of events. When germline blastomeres begin to divide, the nascent mitotic spindle complex rotates by roughly 90° (ref. 17). Before rotation, PIE-1 accumulates at apparently equal levels around each centrosome of the spindle, and PIE-1 staining diminishes in the nucleus, cytoplasm and P

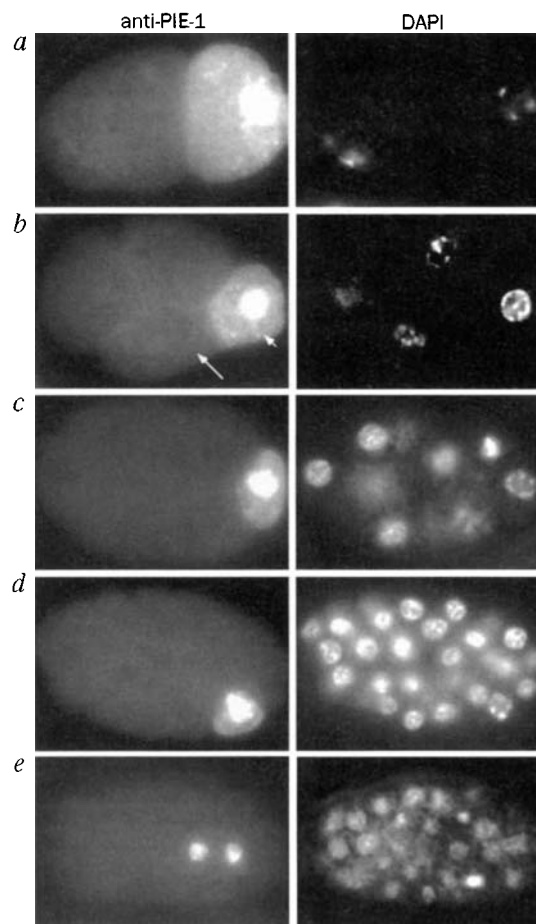


FIG. 3 PIE-1 localization in germline blastomeres. Each row represents a single early embryo stained with an antibody against the PIE-1 protein (left column) or with the dye DAPI to visualize nuclei (right column). a, Late two-cell stage embryo showing PIE-1 in the nucleus and cytoplasm of the P_1 blastomere. Faint, punctate staining in the cytoplasm of P_1 , and all other germline blastomeres shown corresponds to P granules. b, An early four-cell stage embryo with PIE-1 in the nucleus and cytoplasm of the P_2 blastomere (short arrow). Faint PIE-1 staining is detected in the P_2 sister, EMS (long arrow). c, 12-cell stage embryo showing PIE-1 in the P_3 blastomere. d, 46-cell stage showing PIE-1 in the P_4 blastomere. e, 88-cell stage showing PIE-1 in the two P_4 daughters. The P_4 daughters do not divide again during embryogenesis, and in postembryonic development produce only germ cells (sperm and oocytes). PIE-1 is not detected in embryos after roughly 200 min of development. Embryos are 45 μ m in length.

METHODS. The PIE-1 protein sequence shown in Fig. 2a was used to generate three peptides: A (residues 54–73), B (residues 81–105) and C (residues 134–157). Rabbit polyclonal antisera were generated against the individual peptides. Embryos shown were fixed and stained with antisera generated against peptide C, and with DAPI to visualize nuclei, and examined under the fluorescence microscope². Similar staining results were obtained with each of the peptide antisera. PIE-1 staining with the antiserum against peptide C is not detectable in the nucleus, cytoplasm or P granules of embryos from mothers homozygous for the *pie-1* (zu127) mutation.

a
MAQTKPIAEQMAALNNSDDTSFAADRSNLLNATCPARIQNSVDQRKINRSFNDSLSGGY -60
SGKWLRPKREALKITPLAQIDEAPATKRHSASAKDKHTEYKTRLCDAFRREGYCPYNDNCT -120
YAHGQDELVRPRRRQEYYSRDPPRRRRDSRRRDDVDTINRSSSSASKHHDENRRPSNN -180
HGSSNRRQICHNFERNCRYPGRCFIHVEQMQHFNANATVYAPSSDCPPPIAYYHHHP -240
HQQQQFLFFMPYFLAPPQAGQAGFPVQYIPHQHDLNNSQPMYAPMPTYYQPINSN -300
GMPMDVTIDPNATGGAFEVFPDGFSSQPPPTIIS -335

b
PIE-1 Y K T R L C D A F R R E G Y C P Y N D N C T Y A H -123
NUP475 Y K T R L C R T Y S E S G R C R Y G A K Q F A H -120
U2AF35 K Y R P S P F Y N K T G A C R F G N R C C C S R K H -181
su(S) R K L E L C K F Y L M - D C C A K R D K C S Y M H -354
unkem Y K T E P C K R P P R - - L C R Q G Y A C C Q Y H -244

PIE-1 N R R Q I C H N F E R - G N C R Y G P R C R F I H -208
NUP475 Y K T E L C H K F Y L Q G R C F Y G S N H F I H -158
U2AF35 W K V A I C G L F E M - Q K C E K G K H N F L H -321
su(S) H K E F P C K Y Y Y L G M D C Y A G D D L F Y H -379
unkem Y K S T K C N D V Q Q A G Y C F R S V F C A F A H -324

FIG. 2 The *pie-1* locus. a, Deduced amino-acid sequence from the nucleotide sequence of a 1,105-bp *pie-1* cDNA clone. Nucleotides 529 to 746 are coding sequences deleted in *pie-1*(zu127) (data not shown). b, Alignment of the *pie-1* zinc-finger motifs with sequences from nup475 (ref. 4), U2AF35 (ref. 12) and unkempt¹⁴.

METHODS. The spontaneous mutation *pie-1*(zu177::Tc1) was obtained in a previously described genetic screen¹¹. The mutant strain was outcrossed several times and placed over a chromosome marked with *nob-1*(ct230) and *unc-25*(e156) which flank *pie-1*. Recombinant animals yielded a map order of *nob-1*–32–*pie-1*–32–*unc-25*, and in all recombinants a single novel Tc1 transposon co-segregated with the *pie-1* mutation. The *C. elegans* genomic DNA flanking this transposon was isolated as described²⁴. Probes were prepared and used to isolate four full-length cDNA clones; each cDNA contained the trans-spliced leader sequence SL1 (ref. 25) at its 5' end and a poly(A) tail of varying length at its 3' end. Three lines of evidence indicate that the open reading frame encodes the PIE-1 protein: The *pie-1*(zu177::Tc1) and *pie-1*(zu127) mutations are a transposon insertion and a deletion, respectively, in the genomic DNA corresponding to this open reading frame; *in vitro* transcribed RNA from these sequences, prepared as described^{2,26}, can induce a *pie-1* phenocopy when microinjected into wild-type animals; and antibodies raised against peptides deduced from this sequence recognize a protein that is present in wild-type embryos but absent in *pie-1* mutants (Fig. 3 and data not shown).

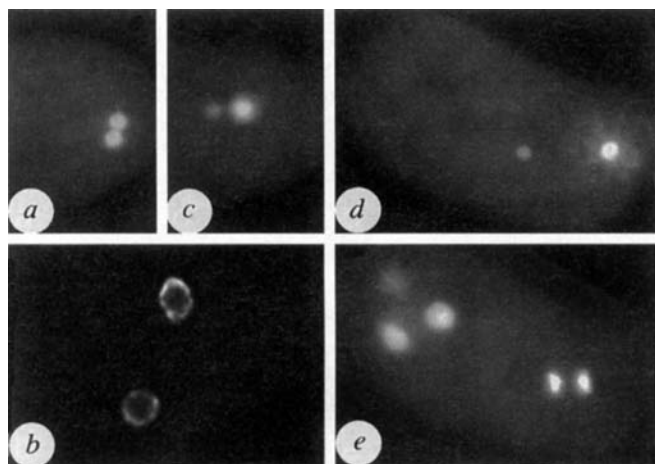


FIG. 4 Centrosomal localization of PIE-1 during division of the germline blastomere P_1 . All images except e are of embryos stained with an affinity-purified antibody against PIE-1 peptide A and viewed with either a standard fluorescence microscope (a, c, d, e) or an optical sectioning microscope (b). a, Two-cell stage embryo (only the P_1 blastomere is shown). P_1 is at prophase; PIE-1 is not detected in the nucleus (not visible) but is detected prominently in the two centrosomes, which are shown here just before rotation of the centrosome-spindle complex (compare with the nuclear staining of the interphase P_1 blastomere in Fig. 3a). b, High-magnification image of PIE-1 staining at the P_1 centrosomes. c, The P_1 blastomere after rotation of the centrosome-spindle complex. The anterior (left) centrosome stains less intensely than the posterior (right) centrosome. d, Division of the AB and P_1 blastomeres (compare with DAPI image shown in e). The mitotic spindles of AB and P_1 have elongated; both centrosomes associated with the P_1 spindle have detectable PIE-1 staining (arrows), although the posterior centrosome (destined for the germline blastomere P_3) shows a much higher level of staining than the anterior centrosome (destined for the somatic blastomere EMS). Neither of the centrosomes associated with the dividing AB blastomere have detectable PIE-1 staining. e, DAPI image of embryo shown in d. The two nascent daughter nuclei of the dividing AB blastomere are visible on the left, and the two nascent daughter nuclei of the dividing P_1 blastomere are visible on the right.

METHODS. Identification of the PIE-1-containing structures shown here as centrosomes is based on co-staining experiments with an antibody against beta-tubulin¹⁷; in such experiments the PIE-1-containing structures are localized at the centre of each spindle aster (data not shown). Embryos were prepared and stained as described in Fig. 3. Image in b was collected on a DeltaVision SA3.1 wide-field deconvolution optical sectioning microscope (Applied Precision). The image was deconvolved using the reiterative constrained method²⁷.

granules (Fig. 4a, b). After spindle rotation, the level of PIE-1 staining in one of the two centrosomes diminishes rapidly and becomes undetectable (Fig. 4c, d). Thus at the completion of cell division, only one daughter cell contains PIE-1 at its centrosome, and that daughter invariably is the new germline blastomere (P_1 , P_2 , P_3 or P_4). When the P_4 blastomere divides, PIE-1 staining persists in both centrosomes (data not shown).

Although we do not know the behaviour of individual PIE-1 molecules during the cell cycle, one model is that the PIE-1 protein translocates to centrosomes at cell division. Other proteins, such as NuMA (ref. 18) and CP190 (ref. 19), have been described previously that seem to cycle between the nucleus and centrosomes. We are not aware of proteins other than PIE-1 that disappear from one of the two centrosomes, and thus become distributed asymmetrically after cell division.

What might distinguish the two centrosomes in a dividing germline blastomere? In a study on the control of spindle orientation in *C. elegans*, the relative positions of the two centrosomes of a germline blastomere were interchanged before rotation of the mitotic spindle was complete²⁰. These manipulated embryos developed normally, suggesting that both centrosomes initially are equivalent. Thus we propose that rotation of the mitotic

spindle in the germline blastomeres may bring the centrosomes into different intracellular environments that affect PIE-1 stability or binding. The idea that asymmetry exists within each germline blastomere is supported by the recent finding that P granules also seem to be degraded or unstable in the pre-somatic 'half' of a germline blastomere before division²¹.

The early divisions of the *C. elegans* embryo represent a classic stem-cell-like lineage in which one pluripotent cell gives rise sequentially to daughters with distinct, and more restricted, developmental potentials. In *C. elegans*, the fate of the first somatic blastomere, AB, is determined in part by responses to external cell signals^{11,22}. As described above, the fate of the second somatic blastomere, EMS, is determined at least in part by two transcription factors that are present in both P_2 and its sister cell EMS^{1,2}. It is likely that differentiation in stem cell lineages in other animals is also determined by several different intercellular or intracellular signals, and that the pluripotent cells must either not contain, or not respond to, these signals.

The PIE-1 protein in *C. elegans* provides an example of how the pluripotent cell in a stem cell lineage might be protected from the signals that promote differentiation in other cells. We have shown here that the PIE-1 protein is localized to the totipotent germline blastomere after each division in the early embryo. This localization correlates with the repression of somatic cell fates³, and also seems to correlate with a general repression of transcription within the germline²³. Thus PIE-1 may represent a localized general repressor that serves to antagonize the activity of a more broadly expressed set of transcriptional activators. This may provide an efficient means for generating diversity within a stem cell lineage: if cells in a stem cell lineage express determinative molecules at different times or in response to different signals, there need not be separate mechanisms for segregating these molecules away from the pluripotent stem cell. Instead, a single mechanism for localizing a general repressor at each division could maintain the pluripotency of the stem cell. □

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CORRESPONDENCE and requests for materials should be addressed to C.C.M. (e-mail: craig.mello@ummed.edu). The cDNA sequence of the pie-1 clone has been deposited in Genbank, accession no. U62896.

Repression of gene expression in the embryonic germ lineage of *C. elegans*

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THE distinction between soma and germline was recognized more than a century ago: somatic cells form the body of an organism, whereas germ cells serve to produce future generations¹. In *Caenorhabditis elegans*, the separation of soma and germline occurs through a series of asymmetrical divisions, in which embryonic germline blastomeres divide unequally to produce one somatic daughter and one germline daughter². Here we show that after each asymmetrical division, embryonically transcribed RNAs are detected in somatic, but not germline, blastomeres. This asymmetry depends on the activity of the germline-specific factor, PIE-1. In the absence of PIE-1, embryonically transcribed RNAs are detected in both somatic and germline blastomeres. Furthermore, ectopic expression of PIE-1 in somatic blastomeres can significantly reduce the accumulation of new transcripts in these cells. Taken together, these results suggest that germ-cell fate depends on an inhibitory mechanism that blocks new gene expression in the early embryonic germ lineage.

To determine whether somatic and germ lineages differ in their capacity for new gene expression, we collected genes transcribed in the early embryo and determined their expression patterns by *in situ* hybridization. An analysis of 16 genes transcribed before the onset of gastrulation (28-cell stage) is shown in Table 1. These genes had been isolated using a variety of different screening methods³⁻¹¹, and are likely to be representative of many RNAs produced during early cleavages. In each case, expression was detected in somatic blastomeres, but not in germline blastomeres. To date, no embryonically transcribed messenger RNAs have been detected in germline blastomeres before the 28-cell stage. Although we have not ruled out the existence of such RNAs, our observations indicate that somatic blastomeres are capable of expressing a large set of genes which remain inactive in the early embryonic germ lineage.

Germline blastomeres divide asymmetrically to give rise to one somatic daughter and one germline daughter (Fig. 1). We found that newly formed somatic blastomeres rapidly acquire the capacity for gene expression. Using a chromosomally integrated, multicopy *pes10::lacZ* transgene⁵, we first detected newly transcribed RNAs in the nuclei of the two somatic blastomeres, ABa and ABp, of three-cell embryos (Fig. 1). In four-cell embryos, activation of the transgene in the somatic blastomere EMS was apparent shortly after completion of the asymmetrical division that generates EMS and its sister, the germline blastomere P₂ (Fig. 1). Similar kinetics of activation were observed for the somatic blastomeres C and D (data not shown). These observations indicate that somatic blastomeres acquire the ability to

transcribe new RNAs minutes after their separation from the germ lineage.

The *pie-1* gene plays an important role in the development of the germ lineage¹². In the absence of maternal *pie-1* activity, descendants of the germline blastomere P₂ adopt fates characteristic of cells descended from the somatic blastomere EMS. As a result, no primordial germ cells are made¹². To study the basis of this transformation, we examined the effects of *pie-1*(-) mutations on the molecular features characteristic of the early

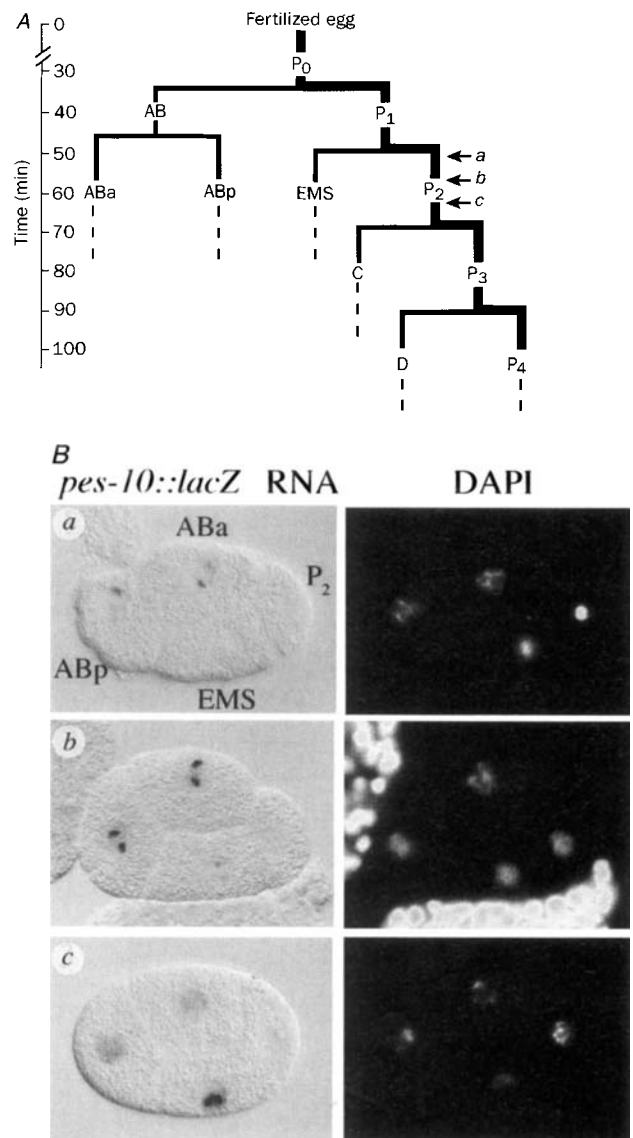


FIG. 1 Soma-specific expression of a *pes-10::lacZ* transgene in four-cell embryos. **A**, Lineage diagram of the first cleavages of the *C. elegans* embryo with the germline shown in bold. Dotted lines represent additional cell divisions. In the 100-cell stage, the germline blastomere P₄ divides symmetrically into the two primordial germ cells Z2 and Z3. These cells do not divide until after hatching. **B**, Four-cell embryos containing a *pes-10::lacZ* transgene were hybridized with an antisense *lacZ* probe (left column) and stained with the DNA-binding dye DAPI (right column), following standard procedures²¹. Embryos **a**, **b** and **c**, were fixed at time points **a**, **b** and **c**, respectively, shown in the lineage diagram in **A** (arrows). **a**, At the beginning of the four-cell stage, expression of the *pes-10::lacZ* transgene is detected in ABa and ABp, but not in EMS and P₂. **b**, In the middle of the four-cell stage, expression of the transgene is detected in EMS (two faint nuclear dots), but not in P₂. **c**, At the end of the four-cell stage, ABa and ABp have begun mitosis. The transgene is expressed at high levels in EMS, but no expression is detectable in P₂.

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TABLE 1 Embryonically transcribed RNAs have been detected in somatic but not germline blastomeres

Gene	Function	Earliest stage of transcript detection	Tissue distribution		Method of isolation	References
			Soma	Germline		
<i>pes-10</i>	Unknown	4-cell stage†	+	—	Promoter trapping	Ref. 3
<i>pes-11::lacZ</i>	Unknown	4-cell stage	+	—		A. Godbey, G.S. & A.F. (unpublished results)
<i>pes-12::lacZ</i>	Unknown	4-cell stage	+	—		
<i>vet-5</i>	Unknown	4-cell stage	+	—	Molecular screen for genes expressed preferentially in pre-gastrulation embryos	Ref. 4; J.P. & W.W. (unpublished results)
<i>vet-6</i>	Unknown	4-cell stage	+	—		
<i>vet-2</i>	Unknown	4-cell stage	+	—		
<i>vet-1</i>	Unknown	4/8-cell stage	+	—		
<i>vet-4</i>	Unknown	4/8-cell stage	+	—		
<i>hsp16-2::gfp::lacZ</i>	HSP16	4/8-cell stage*	+	—	Cloning of genes induced by heat-shock	Ref. 5
<i>hsp16-41::gfp::lacZ</i>	HSP16	4/8-cell stage*	+	—		
<i>nhr-2</i>	Unknown	8-cell stage	+	—	Sequence similarity to nuclear hormone receptors	A. Sluder & G. Ruvkin (personal communication)
<i>cul-1</i>	Cell-cycle regulator	15-cell stage	+	—	Genetics	Ref. 6
<i>cey-1::lacZ</i>	Unknown	15-cell stage	+	—	DNA binding protein	Ref. 7
<i>cm14g5</i>	Histone H1	24-cell stage	+	—	Expressed sequence tag	Ref. 8
<i>CellF::lacZ</i>	Initiation of translation (eIF4-A)	28-cell stage	+	—	Promoter trapping and sequence similarity	Ref. 9; G.S. & A.F. (unpublished results)
<i>xol-1::lacZ</i>	Sex determination/ Dosage compensation	28-cell stage	+	—	Genetics	Refs 10, 11

Transcripts were detected by *in situ* hybridization using standard methods²¹. Lack of expression in the germ lineage was scored in 28-cell and younger embryos, where P_2 and P_3 were identified by their characteristic sizes and positions, and P_4 by staining with a P-granule antibody¹⁴. The expression patterns of *pes-10*, *nhr-2* (*crf-2*), *cul-1* (*lin-19*), and *cm14g5* have been reported⁵. Note that *vet-5*, *hsp16-2::gfp::lacZ*, *hsp16-41::gfp::lacZ*, *cul-1*, *nhr-2*, *cey-1* and *CellF* are expressed in the germ lineage in adults (data not shown). Method of isolation refers to the method used to identify the gene listed, as described in the listed reference.

* See Fig. 3 legend for heat-shock protocol.

† *pes-10::lacZ* RNAs have been detected as early as the 3-cell stage (G.S. and A.F., unpublished results).

germline. Germline blastomeres are known to differ from somatic blastomeres in three aspects of RNA metabolism. First, germline blastomeres protect certain maternal RNAs from the rapid degradation observed in somatic blastomeres³. Second, germline blastomeres contain clusters of poly(A)⁺ RNAs³ associated with a set of germline-specific organelles (P granules)¹³. Third, as shown above, germline blastomeres lack many embryonically transcribed RNAs observed in somatic blastomeres. The first two features were unaffected in *pie-1*(−) embryos: these embryos showed normal patterns of RNA clustering in the germline-specific P granules (Fig. 2A), and normal maintenance of maternal RNA in the germ lineage (Fig. 2B). In contrast, we found that patterns of transcription were profoundly affected by loss of *pie-1* activity: newly transcribed RNAs were detected in the germline blastomeres P_1 , P_2 and P_3 of *pie-1*(−) embryos (Fig. 2C). These results indicate that *pie-1* activity is required to suppress the production of new RNAs in germline blastomeres, but is not required for certain other aspects of germline blastomere identity.

PIE-1 apparently acts during a limited time window in early embryogenesis. The 16 genes that we analysed in wild-type embryos were inactive in the germ lineage from the one-cell stage to at least the 28-cell stage (Table 1). In *pie-1*(−) embryos, newly transcribed RNAs were detected in the germ lineage from the 3-cell to the 15-cell stage (Fig. 3). As in the wild type, no transcription was detected in one- and two-cell *pie-1*(−) embryos. These results indicate that *pie-1* is necessary to repress gene expression from the 3-cell to the 15-cell stage, but that additional *pie-1*-independent mechanisms are operating in earlier stages. *pie-1*-independent mechanisms may also be operating in later stages; the precise point at which PIE-1 and other repression mechanisms cease to function in the germ lineage is not known. Absence of gene expression, however, is not a permanent feature of the germ lineage, as newly transcribed RNAs have been detected in the two daughters of P_4 in the 550-cell embryo⁵.

The *pie-1* gene encodes a putative zinc-finger-containing protein that is localized predominantly to the nuclei of the germline blastomeres P_1 , P_2 , P_3 and P_4 (ref. 14). This localization is

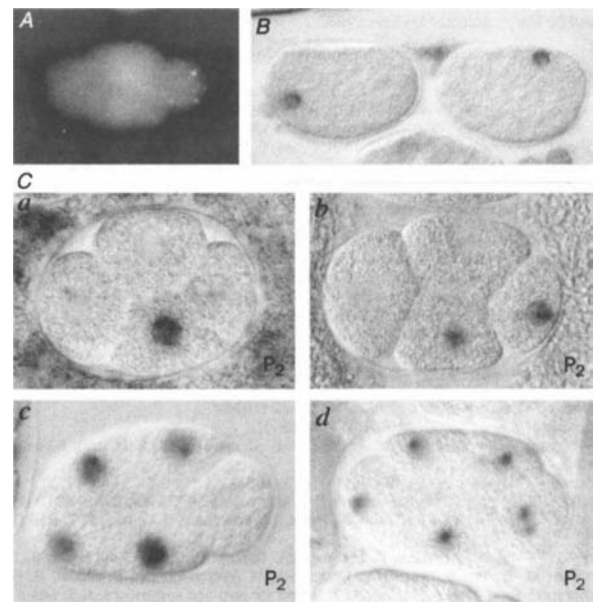
consistent with the possibility that the PIE-1 protein itself acts to repress transcription in germline blastomeres. To address this possibility, we artificially expressed the PIE-1 protein in somatic blastomeres by fusing the *pie-1* coding sequence to the heat-shock promoter *hsp16-41*, and transforming worms with this construct. Under conditions of heat shock, PIE-1 protein is detected in the nuclei of somatic blastomeres as early as the 12-cell stage. We found that such expression is sufficient to reduce significantly the accumulation of newly transcribed RNAs in 15-cell to 50-cell embryos (Fig. 3). In contrast, heat-shock-induced ectopic expression of an unrelated nuclear protein, HLH-1, had no effect on the expression of these RNAs (Fig. 3c). These results indicate that the PIE-1 protein can interfere with gene expression, even in the absence of other germline-specific factors.

Our data demonstrate that *pie-1* activity is both necessary and sufficient to interfere with gene expression in embryonic blastomeres. As *pie-1* is required for the formation of primordial germ cells¹², our findings suggest that *pie-1* activity may promote germline fate by interfering with the production of new mRNAs in germline blastomeres. The mechanism by which the PIE-1 protein interferes with gene expression is not known. The presence of PIE-1 in nuclei¹⁴, and its effects on the accumulation of nuclear RNAs, suggest that PIE-1 functions in the nucleus. PIE-1 may block the production of transcripts directly, by interfering with the transcriptional machinery, or indirectly, for example by causing the immediate degradation of newly transcribed RNAs. In any case, the effect of PIE-1 is quickly reversible during normal development: a PIE-1-positive (germline) blastomere is transcriptionally repressed, whereas its PIE-1-negative (somatic) daughter accumulates new transcripts within minutes of its birth.

These observations raise the question of how repression of gene expression could promote germline fate. Inhibition of transcription might protect germline blastomeres from the activity of transcription factors that would otherwise promote somatic development. One such factor is known: the SKN-1 transcription factor is present in the nuclei of both the somatic blastomere EMS and the germline blastomere P_2 (ref. 15). SKN-1 acts only in EMS

FIG. 2 Distribution of maternal and embryonically transcribed RNAs in wild-type and *pie-1*(-) embryos. A, Embryos derived from *pie-1*(-) hermaphrodites were reacted with an antibody²² against the trimethyl guanosine cap present on P-granule-associated RNA clusters³. RNA clusters are present in the germline blastomere P₂ of a four-cell embryo, as is seen in wild type³. B, Embryos derived from *pie-1*(-) hermaphrodites were hybridized to an antisense *cey-2* probe. *cey-2* maternal RNA is detected predominantly in the germline blastomere P₄ in 28-cell to 50-cell embryos, as is seen in wild type³. C, Embryos derived from *pie-1*(+) (a, c) or *pie-1*(-) (b, d) hermaphrodites were hybridized to probes specific for *pes-10* (a, b) or *hsp16-2::gfp::lacZ* (c, d) RNAs. Embryonically transcribed RNAs are detected in the nucleus of P₂ in *pie-1*(-) but not *pie-1*(+) embryos. Similar results were obtained with *vet-1*, *vet-2*, *vet-4*, *vet-5*, *lin-19*, *pes-11::lacZ*, *pes-12::lacZ*, and *hsp16-41::gfp::lacZ* RNAs. Endogenous RNAs were often detected continuously in germline blastomeres from the 3-cell stage to the 28-cell stage in *pie-1*(-) embryos. In contrast, *lacZ* fusion RNAs were present in P₂ and P₃, but reproducibly absent from P₄, in *pie-1*(-) embryos. As *lacZ* fusion RNAs are more labile than endogenous RNAs³, absence of these RNAs in P₄ in *pie-1*(-) embryos suggests that renewed gene expression in that blastomere may be inhibited by mechanisms not dependent on *pie-1* activity.

METHODS. *pie-1*(zu154) *unc-25*(e156)/qC1 hermaphrodites were transformed with *hsp16-2::gfp::lacZ* or *hsp16-41::gfp::lacZ* and marker *rol-6*(d) DNA²³ to generate extrachromosomal arrays axEx25 and axEx26, respectively. ROL non-UNC [*pie-1*(zu154) *unc-25*(e156)/qC1; axEx25 or 26] and ROL UNC [*pie-1*(zu154) *unc-25*(e156)/*pie-1*(zu154) *unc-25*(e156); axEx25 or 26] gravid hermaphrodites were heat-shocked (15 min, 34 °C), allowed to recover (15–30 min, room temperature), and squashed between slide and coverslip before *in situ* hybridization²¹. This heat-shock regimen was sufficient to induce *gfp::lacZ* RNA in most tissues, including



the adult germline. A lack of heat-shock-induced transcripts in the germ lineage of wild-type embryos was also observed with integrated *hsp16-2::hlh-1* and *hsp16-41::hlh-1* fusions (not shown).

to specify its somatic fate¹⁶, and is repressed from also acting in P₂ by PIE-1 (ref. 12). SKN-1, however, is not the only target of PIE-1 action, as *pie-1*(-); *skn-1*(-) double-mutant embryos still fail to form primordial germ cells¹², and as *pie-1* inhibits the expression of genes that are not regulated by *skn-1* (for example, *pes-10* expression is unaffected in a *skn-1*(-) embryo (data not shown)). We propose that PIE-1 protects germline blastomeres from the activity of determinative transcription factors, including (but not limited to) SKN-1, by generally interfering with the production of new mRNAs in germline blastomeres. Our results are consistent with a general lack of mRNA transcription in

germline blastomeres; nonetheless, it remains possible that certain genes escape PIE-1 regulation.

Analyses of germline development across phyla suggest that germ cells have evolved mechanisms that protect them from the processes that specify the fates of somatic cells¹⁷. Our results suggest that, in *C. elegans*, these mechanisms involve PIE-1-dependent repression of gene expression. An intriguing possibility is that similar mechanisms may be operating in other species where the specification of somatic and germ lineages occurs early. Indeed, a lack of embryonically transcribed RNAs has also been observed in the embryonic germ cells of *Drosophila*^{18–20}. □

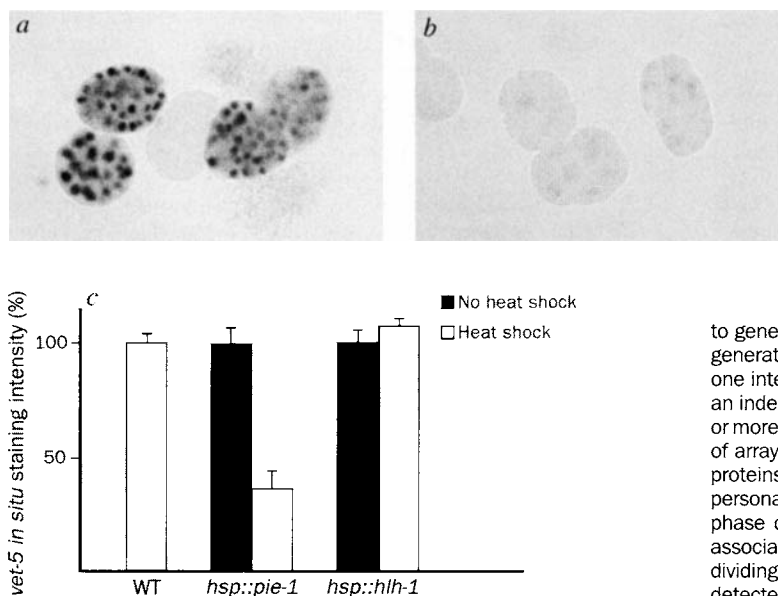


FIG. 3 Somatic expression of PIE-1 can interfere with the accumulation of embryonically transcribed RNAs. Wild-type embryos or embryos transformed with a heat-shock-responsive transgene were incubated for 90 min at 20 °C (no heat shock) or 34 °C (heat shock), allowed to recover for 90 min at room temperature (20–22 °C), and processed for *in situ*

hybridization²¹. a, b, Heat-shocked embryos hybridized to a *vet-5* antisense probe. a, Wild type, b, Embryos transformed with a *hsp::pie-1* fusion. c, Average values for staining intensity were obtained using digitized images of more than 100 15-cell to 28-cell embryos for each genotype and experimental condition. 100% corresponds to levels of staining observed in wild-type embryos after heat shock (no significant difference was seen between heat-shocked and non-heat-shocked wild-type embryos; data not shown). Similar results were obtained with embryonically transcribed RNAs derived from a *pes-11::lacZ* fusion (data not shown).

METHODS. The *pie-1* and *hlh-1* open reading frames were inserted downstream of the *hsp16-41* promoter and injected to generate extrachromosomal arrays. Gamma-ray irradiation was used to generate integrated derivatives of these arrays. Results were derived using one integrated line of *hsp16-41::pie-1*; similar results were obtained with an independent insertion event (not shown). After several generations (10 or more), these strains showed diminished heat-shock effects and evidence of array loss. Heat-shock-induced ectopic expression of PIE-1 and HLH-1 proteins was confirmed using respective antisera (ref. 24 and M. Krause, personal communication). PIE-1 protein was detected in nuclei of interphase cells, and in two dots in metaphase cells, suggesting that PIE-1 associates with centrosomes in dividing somatic blastomeres, as it does in dividing germline blastomeres¹⁴. Occasionally PIE-1 protein was also detected in the cytoplasm of dividing somatic blastomeres. Ectopic HLH-1 protein was predominantly nuclear (M. Krause, personal communication). Heat-shock-induced expression of PIE-1 or HLH-1 lead to embryonic lethality. Embryos that expressed a pulse of PIE-1 protein in somatic lineages during early cleavages developed to the 550-stage and initiated differentiation (intestine-specific granules and muscle twitching), but failed to elongate properly and never hatched (not shown).

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Scavenger receptor-mediated adhesion of microglia to β -amyloid fibrils

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A PATHOLOGICAL hallmark of Alzheimer's disease is the senile plaque, containing β -amyloid fibrils, microglia and astrocytes¹. β -amyloid fibrils exert a cytotoxic effect on neurons², and stimulate microglia to produce neurotoxins, such as reactive oxygen species^{3,4}. Mononuclear phagocytes, including microglia, express scavenger receptors that mediate endocytosis of oxidized low-density lipoproteins⁵, and adhesion to glucose-modified extracellular matrix proteins⁶. Here we report that class A scavenger receptors mediate adhesion of rodent microglia and human monocytes to β -amyloid fibril-coated surfaces leading to secretion of reactive oxygen species and cell immobilization. Thus, class A scavenger receptors are potential therapeutic targets in Alzheimer's disease.

We examined the interactions of microglial class A scavenger receptor (SR) with β -amyloid (A β) fibrils because class A SR contains a collagen-like domain and A β binds to a similar domain on the complement protein C1q and activates C1q (ref. 7), and also because glycated matrix proteins that are ligands for class A SRs are present in Alzheimer's lesions^{8,9}. As microglia associate with A β *in vivo*¹, we examined the adhesion of mouse N9 microglial cells³, primary rat microglia¹⁰, and human monocytes to surfaces coated with both collagen IV (CIV), a matrix protein found in senile plaques¹¹ and A β fibrils (fA β). We compared cell adhesion to fibrillar and non-fibrillar forms of A β 1–42, to A β 25–35, and to

a peptide with a reverse sequence of A β 25–35 (termed rA β 35–25). We used fA β 1–42, an important component of senile plaques¹², and fA β 25–35, because both forms activate microglia and other phagocytes^{3,4}. A β 1–42 and A β 25–35 were in the fibrillar forms as they showed birefringence under polarized light microscopy after staining with Congo red^{2,13}.

Microglia and monocytes do not adhere significantly to CIV-coated surfaces (Fig. 1a and Table 1b). N9 cells adhered to surfaces coated with fA β and CIV in proportion to the amount of fA β deposited. Maximal cell adhesion occurred at ~225 nmol (~1 μ g per spot) of fA β 1–42, and ~235 nmol of fA β 25–35 (Fig. 1a). Similar results were obtained with monocytes cultured for 24 h (not shown). Few N9 cells or monocytes adhered to surfaces coated with CIV and the non-fibrillar form A β 1–42 or to surfaces coated with CIV/rA β 35–25 (Fig. 1a and not shown).

To determine whether residues 25–35 of fA β 1–42 represent a domain to which N9 cells adhere, we pre-incubated N9 cells in medium containing increasing concentrations of fA β 25–35 and then added them to CIV/fA β 1–42-coated surfaces. fA β 25–35 blocked N9 cell adhesion to CIV/fA β 1–42-coated surfaces in a concentration-dependent manner (Fig. 1b) whereas rA β 35–25 did not affect N9 cell adhesion to CIV/fA β 1–42-coated surfaces (Fig. 1b).

SR ligands (for example, acetylated low-density lipoprotein (AcLDL), and maleylated bovine serum albumin (M-BSA)) blocked adhesion of primary rat microglia, N9 cells, and monocytes to surfaces coated with CIV/fA β 25–35 or CIV/fA β 1–42 in a concentration-dependent manner (Table 1a, Fig. 1c and data not shown). Substances that do not bind SRs (such as LDL or BSA), had no effect on microglial adhesion to these surfaces (Table 1a). N9 cells, primary microglia, and monocytes adhered spontaneously to fibronectin- or albumin-coated surfaces but not to CIV-coated surfaces (Table 1a). SR ligands did not inhibit cell adhesion to fibronectin- or albumin-coated surfaces (Table 1a, and data not shown). SR-mediated adhesion of phagocytes occurs in the absence of Ca²⁺ and Mg²⁺ (refs 6, 14). EDTA buffer had no effect on adhesion of N9 cells, primary microglia, or monocytes to surfaces coated with CIV/fA β 1–42 (Table 1b), or CIV/fA β 25–35 (data not shown). As expected, EDTA reduced integrin-mediated adhesion of all three cell types to fibronectin-coated surfaces by ~90% (Table 1b).

Primary rodent and human microglial cells express class A macrophage SR^{10,15,16}. More than 98% of N9 cells stained with 2F8, amonoclonal antibody (mAb) that recognizes murine class A SRs expressed on macrophages and microglia^{14,15}, and endocytosed DiI-labelled AcLDL (not shown). To confirm that class A SRs promote cell adhesion to fA β , COS 7 cells were transfected with complementary DNA¹⁷ encoding bovine type I (COS-SRAI), or type II (COS-SRAII) class A SRs⁶. COS-SRAI and COS-SRAII cells endocytosed roughly seven times more DiI-labelled AcLDL than non-transfected or vector-transfected (COS-vector) COS 7 cells (not shown), confirming expression of functional SRs. COS-SRAI and COS-SRAII cells adhered to CIV/fA β 1–42-coated surfaces, whereas COS-vector cells did not (Fig. 1d). COS-vector, COS-SRAI and COS-SRAII did not adhere to surfaces coated with CIV only (Fig. 1d).

To test whether class A SRs play a significant role in microglial adhesion to fA β , we used anti-SR mAb 2F8 (ref. 14, 15). 2F8 specifically blocked adhesion of N9 cells to CIV/fA β 1–42-coated and to CIV/fA β 25–35-coated surfaces in a dose-dependent fashion (Fig. 1e). This effect was divalent cation independent. MAb 2F8 did not block microglial adhesion to fibronectin-coated surfaces and an isotype-matched (IgG2b) rat mAb did not affect N9 adhesion to fA β -coated surfaces (Fig. 1e). These data confirm that class A SRs are important in the adhesion of microglia to fA β 1–42 and fA β 25–35.

The collagen-like domain of SR is responsible for SR-ligand binding⁵. A synthetic peptide derived from this domain, blocked adhesion of U937 cells expressing SR to glycated CIV (ref. 6). SP1 (with amino-acid sequence PGKPGRSGSPGPKGQK; single-

letter code), a synthetic peptide identical to residues 323–339 of the collagen domain of murine SR, also blocked adhesion of N9 cells or primary microglial cells to CIV/fA β 25–35-coated surfaces in a concentration-dependent fashion (Fig. 2a). In contrast, a 'scrambled' synthetic peptide of identical amino-acid composition did not significantly inhibit adhesion of microglial cells to these surfaces (Fig. 2a).

A β and ligands of macrophage SR added in suspension induce monocytes to secrete reactive oxygen species (ROS)^{3,4,18}. We therefore examined ROS production by microglia adhering to fA β -coated surfaces. N9 cells incubated on CIV/fA β 25–35-coated surfaces produced four times more ROS than N9 cells incubated on CIV-coated surfaces (Fig. 2b). Preincubation of CIV/fA β 25–35-coated surfaces with SP1 blocked fA β -induced ROS secretion

FIG. 1 Adhesion of N9 cells to CIV/fA β -coated surfaces: fA β 25–35 spontaneously formed fibrils in phosphate-buffered saline²⁰, whereas A β 1–42 required a 3-d incubation at 37 °C (ref. 2). **a**, Multisite slides (28.26 mm² per spot) were coated with 2.5 μ g CIV per spot (Fluka), as described⁶, to which fA β 25–35 (■), rA β 35–25 (□), or fA β 1–42 (●) (Bachem Bioscience Inc.), was added as indicated. Phosphate-buffered saline (50 μ l) lacking divalent cations and containing 1 mg ml⁻¹ BSA (PD-BSA) and N9 cells (10⁶ cells ml⁻¹), were added to each spot, incubated for 1 h at 37 °C, and washed 3 times to remove nonadherent cells. Cell adhesion was measured as described²¹ using a fluorescence plate reader (Cytofluor II, Millipore Corp). **b**, N9 cells were incubated for 30 min at 37 °C, in PD-BSA containing various concentrations of fA β 25–35 (■), or rA β 35–25 (●), and 50 μ l of the mixture was added to multisite slides coated with 2.5 μ g CIV per spot and 1 μ g fA β 1–42 per spot: Cell adhesion was quantitated as described above. **c**, N9 cells in PD-BSA (10⁶ cells ml⁻¹) were incubated with various concentrations of AcLDL (●) or M-BSA (■), at 37 °C for 30 min, and added to CIV/fA β 1–42- (solid line) or fibronectin- (dotted line) coated surfaces. **d**, COS 7 cells transfected with SRAI, SRAII, or vector alone, were incubated for 1 h on CIV/fA β 1–42- (shaded bar) or CIV- (hatched bar) coated surfaces. Adherent cells were counted by phase microscopy using a $\times 40$ objective. **e**, N9 microglia were incubated in PD-BSA containing 1 mM Ca²⁺ and 1 mM Mg²⁺ for 30 min with the indicated concentrations of mAb 2F8 or an isotype-matched rat mAb (Zymed) and then for an additional 60 min on surfaces coated with both 2.5 μ g CIV per spot and 1 μ g fA β 1–42 per spot (black bar) or 2.5 μ g CIV per spot/0.5 μ g A β 25–35 per spot (hatched bar). Each data point is the mean of quadruplicate samples from a representative experiment repeated four times. Error bars, s.e.m.

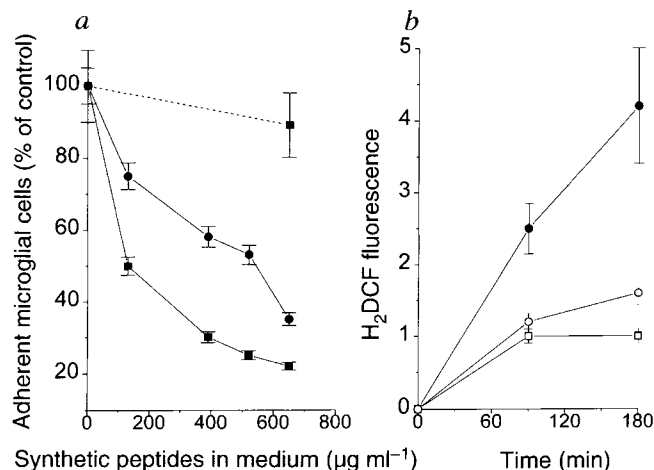
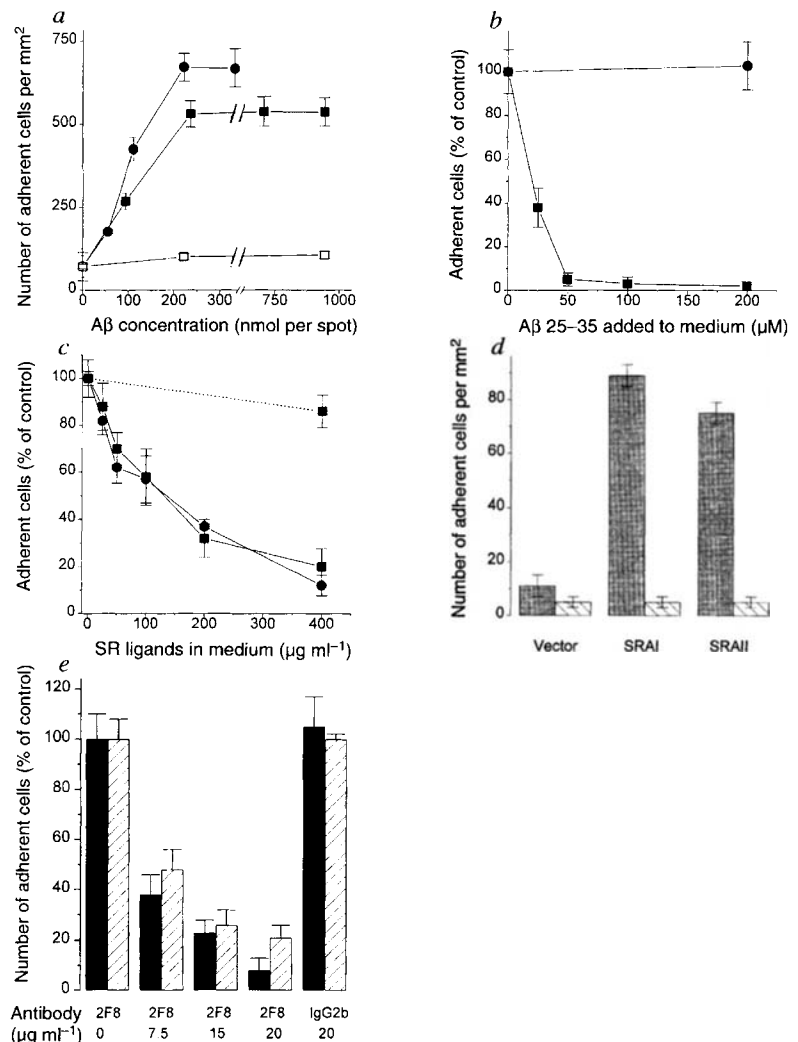


FIG. 2 Effects of a synthetic peptide (SP1) (Quality Controlled Biochemicals) corresponding to residues 323–339 of the collagen-like domain of mouse SR, on microglial-cell adhesion and secretion of ROS. **a**, Primary rat microglia (■) or N9 cells (●) prepared as described¹⁰, were suspended in PD-BSA at 10⁶ cells ml⁻¹. 50 μ l of each suspension was incubated for 1 h at 37 °C with multisite slides coated with both 2.5 μ g CIV and 1 μ g fA β 25–35 per spot in the presence of the indicated amounts of SP1 (solid line) or scrambled peptide (dotted line). Cell adhesion was measured as described in Fig. 1a. **b**, N9 cells (5 $\times 10^4$) in 50 μ l PD-BSA containing 1 μ M H₂DCF, a dye that fluoresces upon oxidation²², were incubated at 37 °C on multisite slides coated with either 2.5 μ g CIV/2 μ g fA β 25–35 per spot or 2.5 μ g CIV alone. N9 cells with or without SP1 on CIV (□); N9 cells without SP1 on CIV/fA β 25–35 (●); N9 cells with SP1 on CIV/fA β 25–35 (○). At the indicated time points aliquots of the medium were taken and fluorescence measured in a fluorescence plate reader (Cytofluor II) as described²². Values are the ratios of H₂DCF fluorescence from N9 cells incubated with CIV/fA β 25–35 compared with CIV alone or N9 cells incubated with CIV/fA β 25–35 plus 1 mg ml⁻¹ SP1 compared with CIV plus 1 mg ml⁻¹ SP1. Each data point is the mean of quadruplicate samples from 2 separate experiments. The experiment was repeated four times with similar results.

TABLE 1 Adhesion of microglia and monocytes to surfaces coated with CIV, CIV/fAβ or fibronectin

a Effect of SR ligands		Number of adherent cells (% of control)		
Surface coated with	Additions	N9 microglia	Primary microglia	Human monocytes
CIV/fAβ1–42	None	100	100	100
	Poly I	8	ND	ND
	Fucoidan	7	ND	14
	AcLDL	22	34	42
	LDL	90	90	ND
	M-BSA	16	ND	11
	BSA	95	99	98
Fibronectin	None	100		
	Poly I	97		
	Fucoidan	96		
	AcLDL	90		
	LDL	98		
	M-BSA	97		
	BSA	99		
b Effect of divalent cations		Number of adherent cells per mm ²		
Surface coated with	Adhesion buffer			
CIV	Ca ²⁺ + Mg ²⁺	18	71	88
CIV	EDTA	35	64	80
Aβ1–42	Ca ²⁺ + Mg ²⁺	800	495	871
Aβ1–42	EDTA	780	442	703
Fibronectin	Ca ²⁺ + Mg ²⁺	1,400	194	477
Fibronectin	EDTA	160	2	49

Multispot slides were coated either with CIV, CIV/fAβ1–42, or with 5 μg fibronectin (see Fig. 1). N9 microglial cells were maintained in culture in RPMI 1640 supplemented with fetal bovine serum. Primary rat microglial cells were isolated from 2–4 d postnatal Sprague-Dawley rat pups and maintained in culture as described¹¹. Over 90% of rat cells were microglia as defined by uptake of Dil-labelled AcLDL and staining with FITC-labelled Ricinus Communis lectin (SIGMA). Human monocytes were prepared as described⁶. a, Cells were suspended at a concentration of 10⁶ ml⁻¹ in PD-BSA and 50 μl of this suspension was added to protein-coated surfaces for 1 h at 37 °C in the presence or absence of the indicated ligands (200 μg ml⁻¹). 100% adhesion of N9 cells, primary microglial cells, and monocytes to CIV/fAβ1–42 is equivalent to 700, 450, and 900 cells per mm², respectively. 100% adhesion of N9 cells to fibronectin is equivalent to 1,120 cells per mm². b, Cells were prepared as above and allowed to adhere to the indicated protein-coated surfaces for 1 h at 37 °C in PD-BSA, containing 1 mM Mg²⁺ and 1 mM Ca²⁺, or 5 mM EDTA. Cell adhesion was measured as described in Fig. 1a. This is a representative experiment repeated three times. Each data point is the mean of triplicate samples whose values are ±10%.

by N9 cells (Fig. 2b), but SP1 had no effect on zymosan-stimulated ROS formation by these cells.

Senile plaques contain a high concentration of microglia that express class A SRs^{1,16}. To determine whether fAβ affect cell movement and/or chemotaxis, we examined migration of primary rat microglia or monocytes across tissue-culture inserts coated with CIV alone or with CIV/fAβ. Without a chemoattractant, 3% of primary microglial cells migrated across CIV-coated inserts, whereas TNFα, a chemoattractant/cytokine produced by microglia³, induced a twofold increase in their migration (Fig. 3). Both spontaneous and TNFα-stimulated migration of primary micro-

glial cells were inhibited almost completely when fAβ25–35 was added to CIV-coated filters (Fig. 3). fAβ25–35 also blocked spontaneous and LTB4-stimulated migration of monocytes across CIV-coated filters (Fig. 3). We used LTB4 because it was a more effective chemoattractant for monocytes than TNFα. These experiments suggest that fAβ may act *in vivo* to retain these cells at sites of Aβ deposition.

SRs are thought to promote adhesion and accumulation of macrophages at sites of deposition of modified plasma and/or extracellular matrix proteins (for example, atherosclerotic lesions)^{6,14,19}, and participate in the production of ROS (ref. 18;

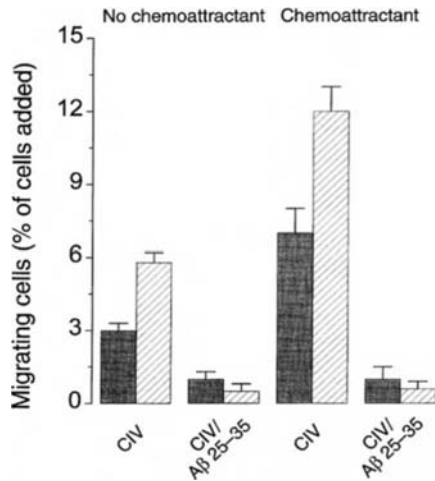


FIG. 3 Effect of fAβ on cell migration. Cell culture inserts (Collaborative Biomedical) were coated with 100 μg of CIV and overlaid with 8 μg of fAβ25–35. 10⁵ primary rat microglia (shaded bars) or 10⁵ human monocytes (hatched bars), were added to the upper chamber of the inserts. TNFα (0.5 × 10⁻⁷ M), the chemoattractant used for rat microglial cells, or LTB4 (0.1 μM) the chemoattractant used for human monocytes, was added to the lower chamber. Inserts were incubated at 37 °C for 10 h, at which time the number of cells that migrated into the lower chamber was determined using a Coulter counter as described²³. Each data point is the mean of duplicate samples from 2 separate experiments. This experiment was repeated a total of 3 times with similar results.

Fig. 2). Whether SRs function in concert with other receptors in initiating these activities remains to be clarified. We propose that deposition of A β fibrils initiates a similar sequence of events in Alzheimer's disease. Microglia adhere to fA β via their class A SR, perhaps in an attempt to clear them from the extracellular milieu. The high density of SR ligands present in these A β deposits causes immobilization of microglia that come into contact with them (Fig. 3), and induces these microglia to secrete cytokines, ROS (Fig. 2 and ref. 4) and nitrogen species that injure neighbouring neurons³. Thus, compounds that block interactions between class A SR and fA β and/or inhibit the release of neurotoxic substances, may be useful in delaying the onset or attenuating the progression of Alzheimer's disease. $\square$

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Functional coupling between ryanodine receptors and L-type calcium channels in neurons

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In skeletal muscle, L-type Ca²⁺ channels act as voltage sensors to control ryanodine-sensitive Ca²⁺ channels in the sarcoplasmic reticulum¹. It has recently been demonstrated that these ryanodine receptors generate a retrograde signal that modifies L-type Ca²⁺-channel activity². Here we demonstrate a tight functional coupling between ryanodine receptors and L-type Ca²⁺ channel in neurons. In cerebellar granule cells, activation of the type-1 metabotropic glutamate receptor (mGluR1) induced a large, oscillating increase of the L-type Ba²⁺ current. Activation occurred independently of inositol 1,4,5-trisphosphate and classical protein kinases, but was mimicked by caffeine and blocked by ryanodine. The kinetics of this blockade were dependent on the frequency of Ba²⁺ current stimulation. Both mGluR1- and caffeine-induced increase in L-type Ca²⁺-channel activity persisted in inside-out membrane patches. In these excised patches, ryanodine suppressed both the mGluR1- and caffeine-activated L-type Ca²⁺ channels. These results demonstrate a novel mechanism for Ca²⁺-channel modulation in neurons.

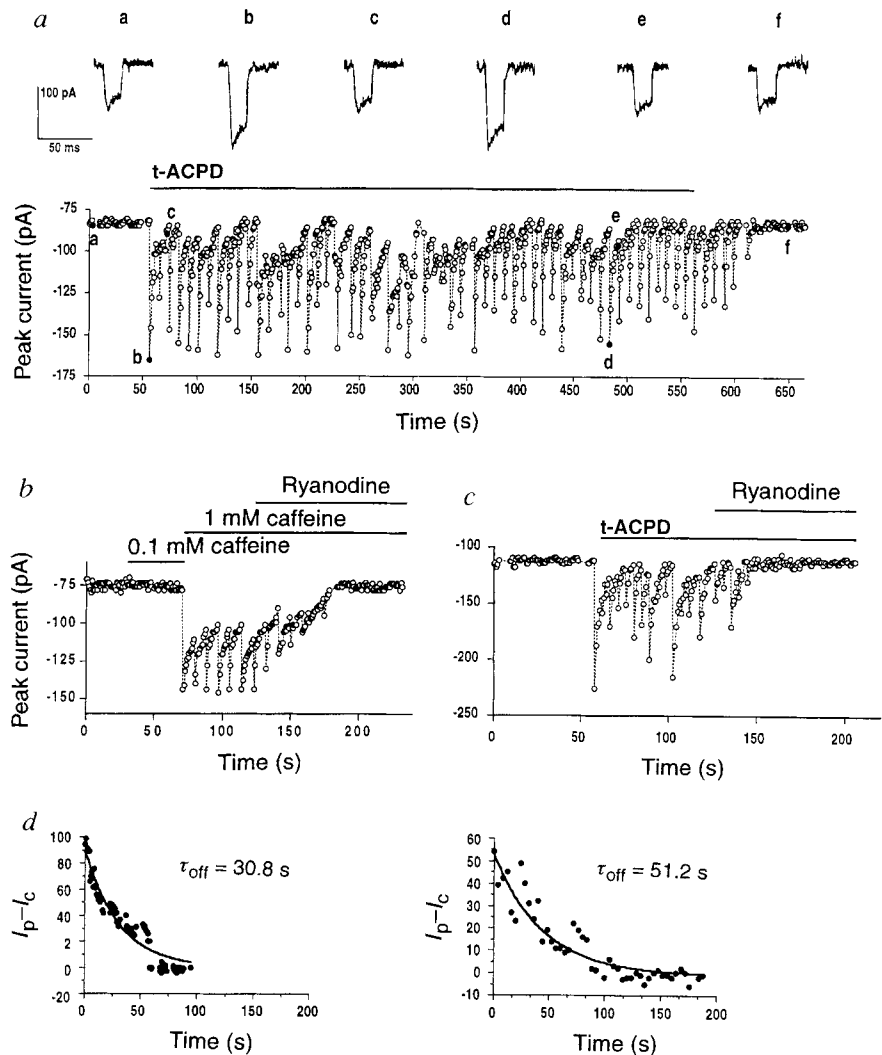
The specific mGluR agonist³ ($\pm$)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (t-ACPD) markedly increased the amplitude of the whole-cell Ba²⁺ current evoked by depolarizing voltage steps ($91.5 \pm 9.3\%$ increase in I_{Ba} in 49 of 76 cells; Fig. 1a). This increase in current followed an oscillating time course that was maintained throughout exposure to t-ACPD, for up to 10 min (Fig. 1a) and during the first minute of wash-out. The current oscillations were not due to an accumulation and removal of voltage-dependent inactivation, because the amplitude of the Ba²⁺ current remained constant during repeated voltage steps applied before agonist application. Exposure to t-ACPD also did not produce a change in the holding current (not shown). Consistent with our previous work^{4,5}, the t-ACPD-induced facilitation was mimicked by the specific mGluR1 agonist quisqualate (5 μ M in the presence of CNQX 50 μ M) and completely blocked by

nimodipine (1 μ M), providing evidence that t-ACPD acted at mGluR1 to increase the current through L-type Ca²⁺ channels. All experiments were done using t-ACPD on cells that were treated with pertussis toxin (PTX) to relieve both mGluR2/3-induced Ca²⁺-channel inhibition⁶ and an agonist-independent tonic inhibition by G_i/G_o (ref. 7). Exposure of non PTX-treated cells to quisqualate (5 μ M in the presence of CNQX 50 μ M) still increased the L-type Ba²⁺ current although this activation was smaller ($39.9 \pm 4.2\%$, $n = 9/14$ cells, not shown). mGluR1 is likely to act through a PTX-insensitive G protein, as the response to t-ACPD was blocked by loading cells with GDP β S (500 μ M, $n = 10/10$, not shown).

The mGluR1-induced facilitation of the L-type Ba²⁺ current was not a general response to phospholipase C (PLC)-coupled receptors, because carbachol, which stimulates the PLC pathway in granule cells by means of a muscarinic receptor⁸, had no effect on the Ba²⁺ current nor did it prevent the stimulation by t-ACPD ($n = 7/7$, not shown). Moreover, signal transduction seems to be independent of InsP₃ and protein kinase C^{9,10}. We found that adding InsP₃ to the patch pipette (100 μ M) did not increase the Ba²⁺ current nor did it prevent facilitation by t-ACPD (data not shown), even though 100 μ M InsP₃ stimulated the Ca²⁺-dependent K⁺ current in the same cells. In addition, neither the inositol 1,4,5-trisphosphate (InsP₃) receptor blocker heparin (100 μ g ml⁻¹, internally applied), nor the specific protein kinase C blocker bisindolylmaleimide GF 109203X (1 μ M, bath applied)¹¹, prevented the facilitation by t-ACPD (the Ba²⁺ current was increased by $91.8 \pm 10.3\%$, $n = 10/15$; $90.5 \pm 8.5\%$, $n = 10/15$ and 92.3 ± 5.2 , $n = 10/16$ cells, respectively; not shown). Finally, neither the protein kinase A inhibitor, PKI (100 μ M in the recording pipette), which we have shown to inhibit cyclic AMP-dependent processes in neurons¹², nor the inhibitor of arachidonic acid production quinacrine (10 μ M, bath applied), prevented the facilitation of the current by t-ACPD ($90.3 \pm 3.1\%$, $n = 12/20$ and 89.6 ± 8.5 , $n = 10/17$ increase respectively; not shown). The action of t-ACPD was not blocked when EGTA (Fig. 1a) was replaced with a high concentration of BAPTA ($91.8 \times 10.4\%$ increase, $n = 15/22$ cells; Fig. 1c). Thus it is unlikely that the global change in Ca²⁺ concentration was the signal generating the Ba²⁺ current oscillations, unless Ca²⁺ stores are spatially localized so that intracellular Ca²⁺ release and reuptake occur in a buffer-inaccessible space.

In cerebellar granule cells, a ryanodine receptor (RyR) antagonist has been shown to block t-ACPD-induced intracellular Ca²⁺ mobilization¹³. We therefore tested whether activation of the RyR might participate in the mGluR1-induced facilitation of Ba²⁺ current. Caffeine, at a concentration (1 mM) which activates the

Fig. 1 Facilitatory coupling between RyRs and Ca^{2+} -channels induced by mGluR1 activation or caffeine. Horizontal bars indicate the time during which the cell was exposed to either t-ACPD (400 μM) or caffeine, or the inhibitor ryanodine (10 μM). *a*, Letters indicate the times at which the top Ba^{2+} -current traces were obtained. *b*, *c*, The recording electrode contained 100 $\mu\text{g ml}^{-1}$ heparin and 20 mM BAPTA. *d*, Representative time course of the absolute peak current amplitude (I_p) subtracted from the mean current control amplitude (I_c) during ryanodine application. The decay was fitted by a monoexponential function: $A\exp(-t/\tau_{\text{off}})$. Ba^{2+} -currents were evoked by depolarizing pulses delivered at 2 Hz frequency (*a*) or 0.25 Hz frequency (*b*). **METHODS.** Mouse cerebellar granule-cell cultures were prepared as described¹⁹ and used at 8–10 d in culture after overnight incubation with PTX (200 ng ml^{-1}). Whole-cell voltage-clamp recordings were made using pipettes with resistances ranging from 5 to 8 $\text{M}\Omega$ when filled with an internal solution containing (in mM): caesium aspartate (120), MgCl_2 (3), HEPES buffer (10), glucose (15), NaCl (16), BAPTA (20) or EGTA (10), Na_2ATP (3) cyclic AMP (1) and adjusted to pH 7.4 with CsOH. The external bathing solution contained (in mM): BaCl_2 (20), HEPES (10), tetraethylammonium chloride (20), sodium gluconate (120), glucose (10) and tetrodotoxin (3×10^{-4}) adjusted to pH 7.4 with NaOH. Similar results were obtained when BaCl_2 was replaced by 10 mM CaCl_2 . The osmolality of all solutions was adjusted to 310–315 mOsm l^{-1} with glucose. Control and drug solutions were applied with a fast gravity-driven perfusion system. Currents were evoked by 50-ms duration voltage pulses from a holding potential of -80 mV to a test potential of -10 mV, delivered at 2 Hz. Current signals were recorded with an axopatch 200 amplifier (Axon Instruments, CA). Data were analysed with the pClamp 6 software (Axon Instruments). All whole-cell recordings were corrected for linear leak and capacity currents. Measurements were made at the peak current. The percentage increase of the Ba^{2+} -current amplitude indicated in the main text was calculated as the ratio of the maximum amplitude reached during oscillations in the presence of agonist to the mean amplitude before adding drug.



RyR, triggered large Ba^{2+} -current oscillations similar to those produced by t-ACPD ($93.6 \pm 7.4\%$ increase, $n = 50/79$; Fig. 1*b*). The effect of caffeine was not caused by an action on cAMP-dependent phosphodiesterases or adenosine receptors, as 1 mM theophylline did not mimic the caffeine effect (data not shown). Moreover, the actions of caffeine and t-ACPD were not additive ($93.7 \pm 7.7\%$ increase in the presence of both agonists, $n = 12/12$, not shown), suggesting that they facilitated the same population of L-type Ca^{2+} channels.

To test whether the transduction mechanism stimulated by mGluR1 involved activation of RyRs, we examined the effects of ryanodine and other inhibitors of RyRs. Ryanodine did not affect the Ba^{2+} current in unstimulated cells (not shown), but progressively inhibited the oscillatory increase in Ba^{2+} current triggered either by caffeine (Fig. 1*b*, $n = 30/33$) or t-ACPD (Fig. 1*c*, $n = 35/39$). The same results were found with two other RyR antagonists, procaine (externally applied, $n = 15/17$) and ruthenium red (5 μM ; internally applied; not shown, $n = 12/14$). These results provided evidence that activation of RyRs was sufficient to induce L-type Ba^{2+} -current facilitation and was required for the mGluR1-induced facilitation of these channels. We also observed a marked use-dependence of the block produced by ryanodine. As shown in Fig. 1*d*, the time course of ryanodine-induced blockade of the Ba^{2+} current was inversely proportional to the frequency of depolarizing voltage steps ($\tau_{\text{off}} = 30.2 \pm 6.4$ s, $n = 35$ and

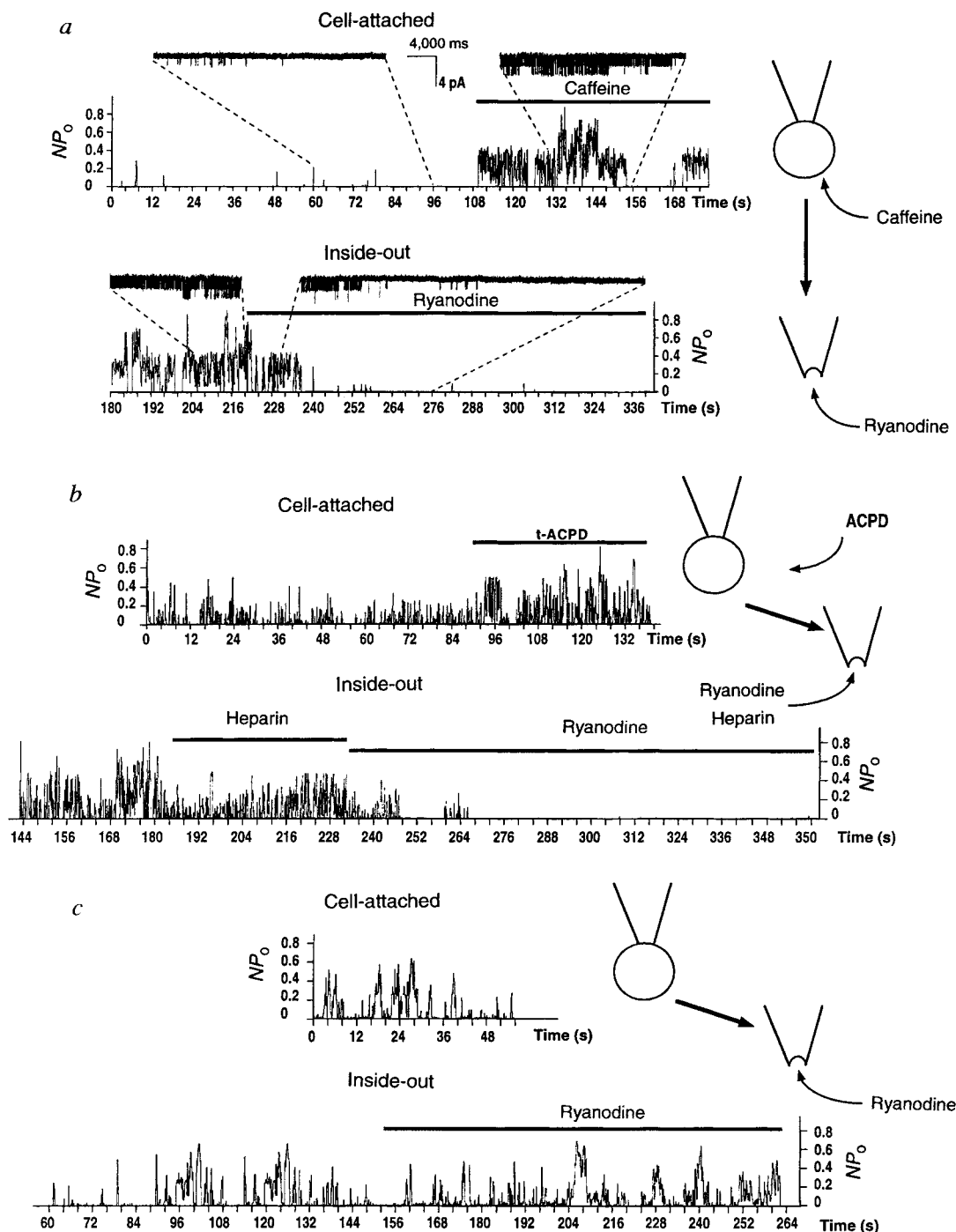
$\tau_{\text{off}} = 61.0 \pm 6.8$ s, $n = 20$ when Ba^{2+} current was induced by a 2 Hz or 0.25 Hz frequency stimulation, respectively). The use-dependence of ryanodine blockade suggests that RyR activity depends critically on coupling to the L-type Ca^{2+} channel.

Although cyclic ADP-ribose has been reported to be an endogenous activator of RyR¹⁴, we found that addition of this compound (2 μM) to the whole-cell recording pipette did not modify the facilitatory effect of t-ACPD on the Ba^{2+} current ($91.5 \pm 5.2\%$ increase, $n = 10/15$, not shown).

The possibility that RyRs interact with L-type Ca^{2+} channels leads us to examine the mechanism of coupling at the single-channel level. A dihydropyridine-sensitive Ca^{2+} channel of 23.5 pS conductance was recorded in cell-attached patches. The channel open-time distribution was best fitted with the sum of two exponentials with time constants $\tau_1 = 2.4 \pm 0.6$ ms and $\tau_2 = 22.4 \pm 7.6$ ms ($n = 33$). These properties were consistent with previous characterizations⁴. Bath application of either caffeine or t-ACPD increased the open probability of these channels by $95 \pm 7\%$ ($n = 12/18$) and $99 \pm 9\%$ ($n = 14/19$), respectively (top histograms in Fig. 2*a*, *b*).

The coupling between RyRs and L-type Ca^{2+} channels seemed to be quite tight, as the facilitation of the Ca^{2+} -channel activity either by caffeine or t-ACPD was maintained after excision of the channels into the inside-out configuration (lower histograms in Fig. 2*a*, *b*, $n = 22/26$). Ryanodine, but not heparin, applied to the

FIG. 2 Tight interaction between RyRs and L-type Ca^{2+} channel induced by RyR or mGluR1 activation. The histograms show Ca^{2+} -channel open probability ($-NP_o$, where N is the putative number of channels in the patch and P_o the open probability of each channel) measured in consecutive intervals of 100 ms, plotted as a function of time. Top of each panel represents recordings from a cell-attached patch before and after exposure to the indicated drugs (caffeine, 1 mM; t-ACPD, 400 μM). Bottom represents channel activity after excision of the patch and exposure to heparin (100 $\mu\text{g ml}^{-1}$) and/or ryanodine (1 μM). *a*, The patch was held at 0 mV membrane potential and displayed two levels of opening (-1.1 pA each, insets). *b*, As *a*, but note the increased Ca^{2+} -channel activity in the presence of t-ACPD. *c*, Lack of effect of ryanodine on L-type Ca^{2+} channels nonstimulated by caffeine or t-ACPD. Definitions as in *a*. **METHODS.** The bathing medium for cell-attached recording contained (in mM): sodium gluconate (120), MgCl_2 (4), HEPES (7.5), glucose (10), tetraethylammonium chloride (20) and TTX (3×10^{-4}) adjusted to pH 7.4 with NaOH. The bathing medium for inside-out recording contained (in mM): aspartic acid (105), HEPES (10), MgCl_2 (3), glucose (15), NaCl (16) and EGTA (8) adjusted to pH 7.4 with CsOH. The pipette solution contained (in mM): BaCl_2 (110), HEPES (10) and Bay K8644 (10^{-3}). The pH was adjusted to 7.4 with NaOH. Unitary currents were filtered at 1 kHz and sampled at 3 kHz. The threshold for detecting opening and closing transitions was set at 50% of the open level.



cytoplasmic surface of these inside-out patches, 40 s to 9.3 min after excision of the patches, abolished the Ca^{2+} -channel activity within 47.7 ± 9.8 s, $n = 21/22$ (Fig. 2b, lower histogram). Ryanodine had no effect on the activity of non-caffeine- or non-t-ACPD-prestimulated Ca^{2+} channels recorded in either cell-attached (not shown, $n = 4/4$) or inside-out patches (Fig. 2c, $n = 7/7$), ruling out a direct blockage of the Ca^{2+} channels by ryanodine. Neither the single-channel conductance nor the open-time constants were affected by drugs or excision of the patch, suggesting that the same population of channels was recruited in all conditions.

Our results suggest that activation of mGluR1 triggers a functional coupling between RyRs and L-type Ca^{2+} channels that produces a cyclical facilitation of the L-type Ca^{2+} channel (Fig. 3c). Transduction by mGluR1 involves a G protein, as it is blocked by GDP β S, but this is likely to occur at a step before coupling

between RyR and L-type Ca^{2+} channel as intracellular GDP β S did not prevent the effect of caffeine (not shown). The mechanism that triggers this functional coupling between RyRs and L-type Ca^{2+} channels is still unknown, but is tight enough to persist after excision of the patch (Fig. 3d). We suggest that, as in skeletal muscle^{1,2}, RyRs and L-type Ca^{2+} channels are capable of bidirectional signalling in neurons. Because ryanodine blocks t-ACPD- or caffeine-stimulated Ca^{2+} channels (Figs 1b, c, 2a, b), but not control activity, we assume that RyRs control the activity of L-type Ca^{2+} channels. The observation of a use-dependent action of ryanodine (Fig. 1d) suggests that the voltage-dependent gating of the L-type Ca^{2+} channel may control the interaction of ryanodine with the RyR. The properties of stimulated Ca^{2+} channels differ from those of the unstimulated L-type Ca^{2+} channels, as ryanodine abolished not only the facilitated Ca^{2+} -channel activity, but

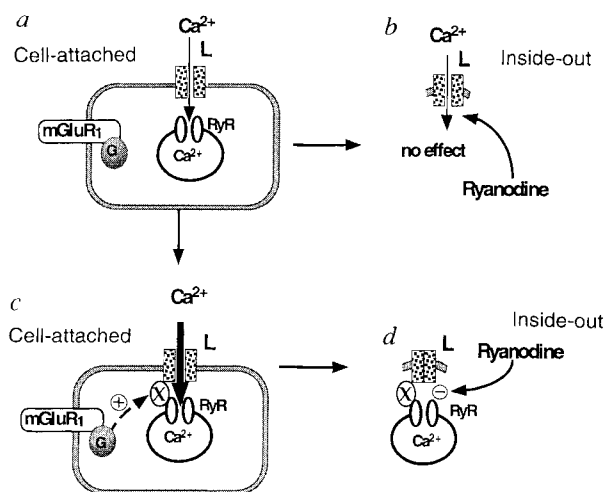


FIG. 3 Model for the coupling between the ryanodine receptor and L-type Ca^{2+} channel induced by mGluR1 stimulation. In unstimulated cells ryanodine has no effect on the Ca^{2+} channel recorded in both cell-attached (a) and inside-out (b) configurations, indicating a lack of interaction between RyR and the membrane Ca^{2+} channels in nonstimulated cells. Stimulation with mGluR1 activates a PTX-insensitive G protein to generate an unidentified second messenger, which in turn triggers a facilitatory interaction between the RyR and the L-type Ca^{2+} channel, leading to a larger transmembrane Ca^{2+} current (c). Although the mechanism by which mGluR1 induces this coupling is unknown, the involvement of a second messenger is likely as t-ACPD applied in the bath activated channels under the cell-attached recording pipette. The facilitatory coupling between the RyR and the L-type Ca^{2+} channel is sufficiently tight to remain in excised patches where ryanodine disrupts this interaction (d). The model does not exclude the possibility that an unidentified intermediate factor (X) links RyR with the L-type Ca^{2+} channel.

all of the single-channel activity (Fig. 2b). An unidentified soluble intracellular factor may participate in the recovery of Ca^{2+} -channel activity after wash-out of t-ACPD, as the mGluR-induced channel stimulation persisted in inside-out patches after washing out agonist (Fig. 2a, b) whereas it returned to control levels in whole-cell experiments (Fig. 1a).

Coupling of RyRs to Ca^{2+} entry through L-type Ca^{2+} channels by mGluR1 may be important in regulating neuronal electrical activity and synaptic plasticity. Indeed, induction of long-term depression in cerebellar Purkinje cells requires, in addition to Na^{+} influx¹⁵, coincident mGluR1 activation¹⁶, Ca^{2+} entry through voltage-sensitive Ca^{2+} channels¹⁷ and Ca^{2+} release from ryanodine-sensitive stores¹⁸. □

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Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene

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Hiv-1 and related viruses require co-receptors, in addition to CD4, to infect target cells. The chemokine receptor CCR-5 (ref. 1) was recently demonstrated to be a co-receptor for macrophage-tropic (M-tropic) HIV-1 strains^{2–6}, and the orphan⁷ receptor LESTR (also called fusin) allows infection by strains adapted for growth in transformed T-cell lines (T-tropic strains). Here we show that a mutant allele of CCR-5 is present at a high frequency in caucasian populations (allele frequency, 0.092), but is absent in black populations from Western and Central Africa and Japanese populations. A 32-base-pair deletion within the coding region results in a frame shift, and generates a non-functional receptor that does not support membrane fusion or infection by macrophage- and dual-tropic HIV-1 strains. In a cohort of HIV-1-infected caucasian subjects, no individual homozygous for the mutation was found, and the frequency of heterozygotes was 35% lower than in the general population. White blood cells from an individual homozygous for the null allele were found to be highly resistant to infection by M-tropic HIV-1 viruses, confirming that CCR-5 is the major co-receptor for primary HIV-1 strains. The lower frequency of heterozygotes in seropositive patients may indicate partial resistance.

Few molecules have attracted as much immediate attention as the recently cloned and characterized CC-chemokine receptor-5 (CCR-5)¹. CCR-5 was shown to respond to macrophage inflammatory protein (MIP)-1 α , MIP-1 β and RANTES (for regulation-upon-activation, normal T expressed and secreted), the three chemokines identified as the major HIV-suppressive factors produced by CD8⁺ T cells⁸, and released in higher amounts by CD4⁺ T lymphocytes from uninfected but multiply exposed individuals⁹. It has been shown that CCR-5 represents the major co-receptor for primary M-tropic HIV-1 strains^{2–6}. M-tropic strains predominate during the asymptomatic phase of the disease in infected individuals, and are thought to cause HIV-1 transmission. In some individuals, T-tropic viruses capable of replicating

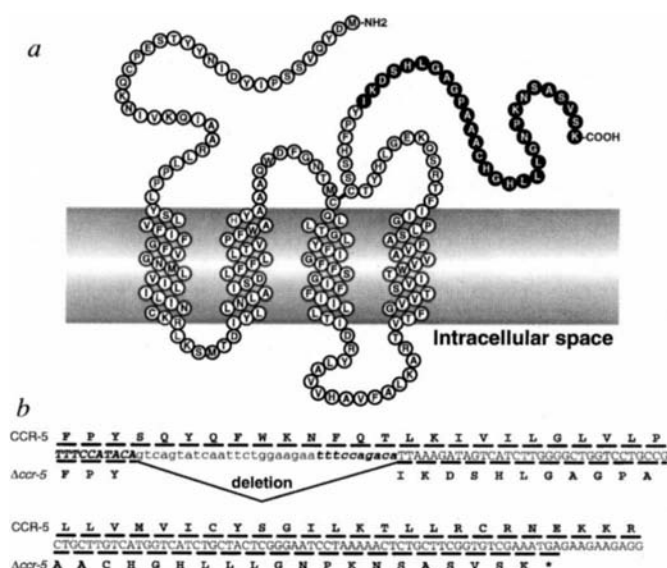


FIG. 1 Structure of the mutant form of human CCR-5. *a*, The amino-acid sequence of the non-functional Δ CCR-5 protein. The transmembrane organization is given by analogy with the predicted transmembrane structure of the wild-type CCR-5, although the correct maturation of the mutant protein up to the plasma membrane has not been demonstrated. Amino acids represented in black correspond to unnatural residues resulting from the frame shift caused by the deletion. The mutant protein lacks the last three transmembrane segments of CCR-5, as well as the regions involved in G-protein-coupling. *b*, Nucleotide sequence of the CCR-5 gene surrounding the deleted region, and translation into the normal receptor (top) or the truncated mutant (Δ CCR-5, bottom). The 10-bp direct repeat is represented in bold italics.

in transformed T-cell lines emerge with time. These virus strains use LESTR (or fusin) as a co-receptor⁷, an orphan receptor also belonging to the chemokine receptor family, but not yet characterized functionally¹⁰⁻¹². Dual-tropic viruses, which may represent transitional forms of the virus in late stages of infection¹³, were shown to use both CCR-5 and LESTR as co-receptors, as well as the CC-chemokine receptors CCR-2b and CCR-3 (ref. 4). The broad spectrum of co-receptor usage of dual-tropic viruses suggests that, within infected individuals, the virus may evolve at least in part from selection by a variety of co-receptors expressed on different cell types.

Some individuals remain uninfected despite repeated exposure to HIV-1 (refs 9, 14, 15). In some of these exposed-uninfected individuals, the reason is the relatively low risk of contamination after a single contact with the virus, but it has been postulated that truly resistant individuals do exist. Indeed, CD4⁺ lymphocytes isolated from exposed-uninfected individuals are highly resistant to infection by primary M-tropic, but not T-tropic, HIV-1 strains. Also, peripheral blood mononuclear cells (PBMCs) from different donors are not infected equally with various HIV-1 strains¹⁶⁻¹⁸. Given the importance of CCR-5 in the fusion event that mediates infection by M-tropic viruses, we postulated that variants of CCR-5 could be responsible for the relative

or absolute resistance to HIV-1 infection exhibited by some individuals, and possibly also for the variability of disease progression in infected patients¹⁹. To test this hypothesis, we selected three HIV-1 infected patients in which disease progression is slow, and four seronegative individuals as controls; the full coding region of their CCR-5 gene was amplified by the polymerase chain reaction (PCR) and sequenced. To our surprise, one of the slow progressors, but also two of the uninfected controls, exhibited heterozygosity at the CCR-5 locus for a biallelic polymorphism. The frequent allele corresponded to the published CCR-5 sequence¹, and the minor one displayed a 32-bp deletion within the coding sequence, in a region corresponding to the second extracellular loop of the receptor (Fig. 1). Cloning of the PCR product and sequencing of several clones confirmed the deletion. The deletion causes a frame shift, which is expected to result in premature termination of translation. The protein encoded by this mutant allele (Δ CCR-5) therefore lacks the last three transmembrane segments of the receptor. A 10-bp direct repeat flanking the deleted region (Fig. 1b) on both sides is expected to have promoted the recombination event leading to the deletion. Numerous mutagenesis studies performed on various classes of G-protein-coupled receptors, including chemokine receptors, show that such a truncated protein is not functional in terms of chemokine-induced signal transduction: it lacks the third intracellular loop and carboxy-terminal cytoplasmic domains, the two regions involved primarily in G-protein coupling²⁰. To test whether the truncated protein was able to function as an HIV-1 co-receptor, we tested its ability to support membrane fusion by both primary M-tropic and dual-tropic virus env proteins. The recombinant protein was expressed in quail QT6 cells together with human CD4. The QT6 cells were then mixed with HeLa cells expressing the indicated viral env protein, and the extent of cell-cell fusion was measured using a sensitive and quantitative gene-reporter assay. In contrast to wild-type CCR-5, the truncated receptor did not allow fusion with cells expressing the env protein from either M-tropic or dual-tropic viruses (Fig. 2a). Coexpression of Δ CCR-5 with wild-type CCR-5 reduced the efficiency of fusion for both JR-FL and 89.6 envelopes in three independent experiments. In comparison, expression of the Duffy chemokine receptor, which does not function as an HIV co-receptor, with wild-type CCR-5 had no effect on fusion efficiency. Whether this *in vitro* inhibitory effect also occurs *in vivo* is not yet known. We also found that Δ CCR-5 failed to support infection by either JR-FL or 89.6 (Fig. 2b). Thus Δ CCR-5 fails to support either membrane fusion or virus infection.

Based on the 14 chromosomes tested in the first experiment, the

TABLE 1 Genotype and allele frequencies of CCR-5 and Δ CCR-5 in cohorts of Caucasians

	Seronegative			Seropositive			χ^2
	Number	Frequency	s.e.	Number	Frequency	s.e.	
Genotypes							
CCR-5/CCR-5	582	0.827	0.014	645	0.892	0.012	1 degree of freedom
CCR-5/ Δ CCR-5	114	0.162	0.014	78	0.108	0.012	12.7
Δ CCR-5/ Δ CCR-5	8	0.011	0.004	0	0.000	<0.001	$P < 0.0005$
Total	704	1.000		723	1.000		
Alleles							1 degree of freedom
CCR-5	1278	0.908	0.008	1368	0.946	0.006	15.1
Δ CCR-5	130	0.092	0.008	78	0.054	0.006	$P < 0.0005$
Total	1408	1.000		1446	1.000		

Genotype and allele frequencies for CCR-5 and Δ CCR-5 in seronegative and seropositive cohorts of Caucasians. Standard errors of the genotype and allelic frequency estimates were calculated as $\sqrt{P(1-P)/n}$. The standard error for the Δ CCR-5/ Δ CCR-5 genotype in the seropositive cohort (no homozygotes found) was assumed to be smaller than if a single individual with this genotype was observed. Genotype and allelic frequencies in seronegative and seropositive samples were compared by 2×2 contingency tables yielding χ^2 with 1 degree of freedom. CCR-5/ Δ CCR-5 heterozygous and Δ CCR-5/ Δ CCR-5 homozygous classes were pooled when comparing genotype frequencies to account for the expected values <5 in the homozygous classes. For the origin of samples, see text.

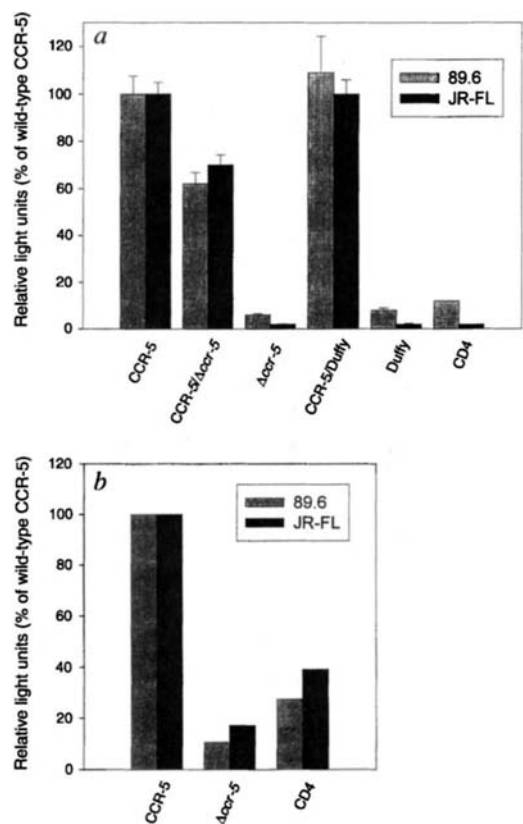


FIG. 2 *a*, Quantification of env protein-mediated fusion by Luciferase assay. To quantify cell-cell fusion events, a modified version of the gene reporter fusion assay²³ was used. Japanese quail QT6 fibrosarcoma cells were transfected or cotransfected as indicated with the pcDNA3 vector (Invitrogen) containing the coding sequence for wild-type CCR-5, the truncated Δ CCR-5 mutant or the Duffy chemokine receptors, or with the pcDNA3 vector alone. The target cells were also transfected with human CD4 expressed from the CMV promoter and the luciferase gene under the control of the T7 promoter. HeLa effector cells were infected (multiplicity of infection = 10) with vaccinia vectors expressing T7-polymerase (vTF1.1) and either the JR-FL (vCB28) or 89.6 (vBD3) envelope proteins. The Luciferase activity resulting from cell fusion is expressed as the percentage of the activity (in relative light units) obtained for wild-type CCR-5. Error bars indicate s.d. within a single experiment (each point was performed in triplicate). Similar results were obtained in two additional experiments. *b*, Quail QT6 cells were transfected with plasmids encoding human CD4, wild-type CCR-5 or Δ CCR-5, and LTR-Luciferase²⁴. Two days later, cells were infected with equivalent amounts of JR-FL and 89.6 (as judged by viral p24), and three days after infection the cells were lysed and Luciferase activity assayed as above. Similar results have been obtained in three independent experiments.

deleted Δ CCR-5 allele was rather frequent in the caucasian population. A more accurate frequency was estimated by testing (Fig. 3) a large cohort of caucasian individuals, including unrelated members of the CEPH (Centre d'Etude des Polymorphismes Humains) families, some members of the IRIBHN staff, and a bank of anonymous DNA samples from healthy individuals collected by the Genetics Department of the Erasme Hospital in Brussels. From a total of more than 700 healthy individuals, the allele frequencies were found to be 0.908 for the wild-type allele, and 0.092 for the mutant allele (Table 1). The genotype frequencies observed in the population were not significantly different from the expected Hardy-Weinberg distribution ($CCR5/CCR5$, 0.827 versus 0.824; $CCR5/\Delta$ CCR-5, 0.162 versus 0.167; Δ CCR-5/ Δ CCR-5, 0.011 versus 0.008; ($\chi^2 = 0.81$; $P < 0.5$), suggesting that the null allele has no drastic effect on fitness. It was confirmed using two CEPH families that the wild-type $CCR5$ gene and its Δ CCR-5 variant were allelic, and segregated in a normal mendelian fashion (data not shown). Cohorts of 124 DNA samples originating from Western and Central Africa (collected from Zaire, Burkina Fasso, Cameroun, Senegal and Benin) and 248 samples of Japanes origin did not reveal a single Δ CCR-5 mutant allele, suggesting that this allele is either absent or extremely rare in Africa and Japan.

The consequences of the existence of a null allele of $CCR5$ in the normal caucasian population were then considered in terms of susceptibility to infection by HIV-1. If, as predicted, $CCR5$ plays a major (not redundant) role in the entry of most primary virus strains into cells, then Δ CCR-5/ Δ CCR-5 individuals should be particularly resistant to HIV-1 challenge, both *in vitro* and *in vivo*. The frequency of the Δ CCR-5/ Δ CCR-5 genotype should therefore be significantly lower in HIV-1 infected patients, and increased in exposed-uninfected individuals. Also, if heterozygotes have a statistical advantage owing to either the lower number of functional receptors on their white blood cells or to possible dominant-negative properties of the mutant allele, the frequency of heterozygotes (and mutant alleles) should be decreased in HIV-infected

populations. These hypotheses were tested by genotyping a large number of seropositive caucasian individuals ($n = 723$) belonging to cohorts originating from various hospitals of major Belgian cities and Paris (Table 1). Within this large series, the frequency of the null Δ CCR-5 allele was significantly reduced from 0.092 to 0.054 ($\chi^2_1 = 15.1$; $P < 0.0005$; see Table 1). This reduction in the Δ CCR-5 allele frequency in the seropositive cohort was due both to a reduction in the frequency of $CCR5/\Delta$ CCR-5 heterozygotes (0.108 versus 0.162) and Δ CCR-5/ Δ CCR-5 homozygotes (0 versus 0.01) ($\chi^2_1 = 12.7$; $P < 0.0005$; Table 1). Great care was taken to include in both the HIV+ and HIV- caucasian cohorts only individuals with a similar geographic origin and with European patronymes. This makes it extremely unlikely that the observed differences in genotype frequencies could result from a different genetic background rather than a true difference in susceptibility to infection by HIV.

TABLE 2 Infection of PBMC from Δ CCR-5/ Δ CCR-5, $CCR5/\Delta$ CCR-5 and $CCR5/CCR5$ individuals with HIV-1 strains

	BAL	JR-FL	SF162	89.6	IIIB
Δ CCR-5/ Δ CCR-5	0.112	0	0	>22.3	>22.3
	0.104	0	0	>22.3	>22.3
$CCR5/CCR5$, $CR5/\Delta$ CCR-5	>22.3	>22.3	>22.3	>22.3	>22.3
	>22.3	>22.3	>22.3	>22.3	>22.3

M-tropic (BAL, JR-FL, SF162), dual-tropic (89.6) and T-tropic (IIIB) HIV-1 strains were used to infect PBMCs prepared from 1 Δ CCR-5/ Δ CCR-5, 3 $CR5/\Delta$ CCR-5 and 2 $CCR5/CCR5$ individuals. p24 antigen (ng ml⁻¹) was assayed 7 days after infection. Most of the measurements were off-scale for the investigated range (>22.3 ng ml⁻¹). The values represent duplicate infections for each condition. All samples derived from $CR5/\Delta$ CCR-5 and $CCR5/CCR5$ individuals gave similar off-scale results with all strains, and are therefore represented together.

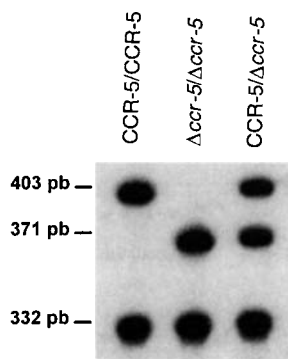


FIG. 3 Genotyping of individuals by PCR. Autoradiography illustrating the pattern resulting from PCR amplification and *EcoRI* cleavage for individuals homozygous for the wild-type *CCR-5* allele (*CCR-5/CCR-5*), the null Δ *CCR-5* allele (Δ *CCR-5/ΔCCR-5*), and for heterozygotes (*CCR-5/ΔCCR-5*). A 735-bp PCR product is cleaved into a common band of 332 bp for both alleles, and into 403 and 371 bp bands for the wild-type and mutant alleles, respectively.

Taken together, the functional and statistical data suggest that *CCR-5* is indeed the major co-receptor responsible for natural infection by M-tropic HIV-1 strains. However, the severely truncated Δ *CCR-5* protein cannot function as an HIV-1 co-receptor (Fig. 2a, b, Table 2). This finding, coupled with the epidemiological data (Table 1), strongly suggests that individuals homozygous for the null Δ *CCR-5* allele (about 1% of the caucasian population) will be highly resistant to HIV-1 infection. It is unclear at this point whether resistance to HIV-1 is absolute or relative, and whether resistance will vary depending on the mode of viral transmission. Larger cohorts of seropositive individuals will have to be tested to clarify this point. Assuming that the genotypic frequencies in both cohorts are true estimates (Table 1), the relative risk of *CCR-5/ΔCCR-5* and Δ *CCR-5/ΔCCR-5* versus *CCR-5/CCR-5* individuals is 0.625 and 0, respectively. Both a decrease in functional *CCR-5* number, and a dominant-negative effect of Δ *CCR-5* *in vivo* comparable to that suggested from our *in vitro* experiments (Fig. 2a), are possible explanations for this relative protection of heterozygotes. The mutant allele, which can be regarded as a natural knock-out in humans, is not accompanied by an obvious phenotype in homozygous individuals. This may be due to the documented redundancy of chemokines and their receptors²¹. Future studies will reveal whether subtle correlations can be found with disease states, especially inflammatory, immune or infectious diseases, given the predominant role of chemokine receptors in the recruitment of white blood cells towards the sites of inflammation^{21,22}. Nevertheless, the lack of overt phenotype of homozygotes, together with the relative protection that characterizes heterozygous subjects, suggests that pharmacological agents that selectively block the ability of HIV-1 to use *CCR-5* as a cofactor could be effective in preventing HIV-1 infection, and would be predicted not to be associated with major side effects resulting from *CCR-5* inactivation. Whether these agents will be effective in delaying the transition of seropositive patients towards AIDS is more questionable, considering the wide range of chemokine receptors used as co-receptors by some HIV-1 primary isolates⁴.

The relatively high frequency of the null allele in the caucasian population could be the result of genetic drift or some undefined selective advantage it may have conferred to its carriers in the distant past. It will be important to search for additional polymorphisms in *CCR-5* and other entry cofactors among diverse populations, as this may help explain different clinical outcomes, such as the exposed-uninfected phenotype, and long-term survivors

Methods

CCR-5 gene amplification and sequencing. The full-size coding region of the *CCR-5* gene was amplified by PCR, using 5'-TCGAGGATCCAAGTGGATTAT-CAAGT-3' and 5'-CTGATCTAGAGCCATGTGCACAACTCT-3' as forward and reverse primers, respectively. The PCR products were sequenced on both strands using the same oligonucleotides as primers, as well as internal primers, and fluorochrome-labelled dideoxynucleotides as terminators. The sequencing products were run on an Applied Biosystem sequencer, and ambiguous positions were searched along the coding sequence. When the presence of a deletion was suspected from direct sequencing, the PCR products were cloned after restriction with *Bam*HI and *Xba*I endonucleases into pCDNA3. Several clones were sequenced to confirm the deletion. The deletion was identical in three unrelated individuals investigated by sequencing.

Fusion and infection assays. Details concerning plasmid and viral vectors have been previously described⁴. All transfections were performed with an identical quantity of plasmid DNA using pCDNA3 as carrier when necessary. To initiate fusion, target and effector cells were mixed in 24-well plates at 37 °C in the presence of ara-C and rifampicin, and allowed to fuse for 8 h. Cells were lysed in 150 μ l of reporter lysis buffer (Promega) and assayed for Luciferase activity according to the manufacturer's instructions (Promega). The LTR-Luciferase infection assay (Fig. 2b) was performed as described²⁴.

Genotyping of individuals by PCR. PCRs were performed on genomic DNA samples, using 5'-CCTGGCTGTGCTCCATGCTG-3' and 5'-CTGATCTAGAGCCATGTGCACAACTCT-3' as forward and reverse primers, respectively. Reaction mixtures consisted of 30 μ l 10 mM Tris-HCl buffer, pH 8.0, containing 50 mM KCl, 0.75 mM MgCl₂, 0.2 mM dCTP, dGTP and dTTP, 0.1 mM dATP, 0.5 μ Ci [α -³²P]-dATP, 0.01% gelatin, 5% DMSO, 200 ng target DNA, 60 ng of each of the primers and 1.5 U Taq polymerase. PCR conditions were: 93 °C for 2 min 30 s, 93 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min; 30 cycles; 72 °C for 6 min. After the PCR reaction, samples were incubated for 60 min at 37 °C with 10 U *Eco*RI, and 2 μ l of the denatured reaction mixture was applied onto a denaturing 5% polyacrylamide gel containing 35% formamide and 5.6 M urea. Bands were detected by autoradiography.

Statistical analysis. Assume that p_1 , p_2 , p_3 and p'_1 , p'_2 , p'_3 are the genotype frequencies of *CCR5/CCR5*, *CCR5/ΔCCR5*, Δ *CCR5/ΔCCR5* individuals in HIV- and HIV+ cohorts, respectively; that x_1 , x_2 , x_3 are the probabilities to become HIV+ conditional on *CCR5/CCR5*, *CCR5/ΔCCR5* and Δ *CCR5/ΔCCR5* genotypes, respectively. The relative risk for *CCR5/ΔCCR5* and Δ *CCR5/ΔCCR5* to contract the disease compared with *CCR5/CCR5* individuals was computed as x_2/x_1 and x_3/x_1 , respectively from $p'_1 = (p_1 * x_1) / (p_1 * x_1 + p_2 * x_2 + p_3 * x_3)$; $p'_2 = (p_2 * x_2) / (p_1 * x_1 + p_2 * x_2 + p_3 * x_3)$; $p'_3 = (p_3 * x_3) / (p_1 * x_1 + p_2 * x_2 + p_3 * x_3)$.

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CORRESPONDENCE and requests for materials should be addressed to M.P. (e-mail: mparment@ulb.ac.be). The nucleotide sequence of the mutant Δ *CCR-5* allele has been deposited in the Genbank/EMBL database, accession no. X93933.

Specific cytotoxic T cells eliminate cells producing neutralizing antibodies

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In medically important infections with cytopathic viruses, neutralizing antibodies are generated within 6–14 days. In contrast, such protective antibodies appear late (50–150 days) after infection with immunodeficiency virus (HIV) and hepatitis B virus (HBV) in humans, or lymphocytic choriomeningitis virus (LCMV) in mice^{1–6}. However, during these infections, non-neutralizing antibodies appear much earlier^{2,6,7}. It has been proposed that T cells suppress antibody responses generally and against viruses *in vitro*^{6,8–10}. Here we show that the suppression of neutralizing-antibody responses in LCMV infections in mice is due to selective infection of neutralizing-antibody-producing B cells by this non-cytopathic virus, and their subsequent destruction by virus-specific cytotoxic T cells. Such specific B-cell elimination that leads to a delay in neutralizing-antibody production could help to establish persistent virus infections by non-cytopathic viruses.

LCMV is a non-cytopathic virus with a wide tropism in its natural murine host, which includes antigen-presenting cells like macrophages and dendritic cells and most other somatic cells. Recovery from primary infection depends on CD8⁺ T cells and perforin and protection against reinfection is mediated by CD8⁺ T cells and by neutralizing antibodies^{5,6,11,12}. LCMV infection leads to immunosuppression due to immunopathological destruction of antigen-presenting cells by virus-specific CD8⁺ T cells^{13–15}. Although neutralizing-antibody responses directed against the LCMV surface glycoprotein (GP) are suppressed, non-neutralizing antibody responses specific for the nucleoprotein (NP) of LCMV are usually unimpaired⁶.

To explore the basis of this selective suppression, we tested whether B cells producing LCMV-neutralizing antibodies were infected preferentially by the virus and were therefore susceptible to lysis by LCMV-specific cytotoxic T cells. Neutralizing antibody titres are not—or are only rarely—measurable after infection with a low dose (10² plaque-forming units (PFU)) of LCMV isolate WE (LCMV-WE) for more than 100 days after infection, but are detectable after day 50 in mice infected with a high dose (10⁶ PFU) of LCMV-WE (Fig. 1a, b). When mice were treated with an anti-CD8-specific T-cell-depleting monoclonal antibody, neutralizing anti-LCMV antibodies were detected earlier (by day 25) after low- and high-dose LCMV infection (Fig. 1c, and data not shown)⁶. In contrast, non-neutralizing antibodies specific for the protein of viral NP appeared early (by 6–8 days after infection) and kinetics were comparable for normal and CD8⁺ T-cell-depleted mice (Fig. 1a–c, and data not shown).

To investigate further the humoral immune response against LCMV at a single-cell level *ex vivo*, hybridomas were generated by fusing spleen cells of either normal or CD8⁺ T-cell-depleted mice on days 2, 4, 6, 10 and 25 after infection with LCMV-WE. Although neutralizing antibodies were not detectable in the serum, we were able to generate hybridomas secreting NP-specific antibodies detected by an NP-specific enzyme-linked immunosorbent assay (ELISA)⁶, but also neutralizing-antibody-producing hybridomas on day 4 after infection of both normal and CD8⁺ T-cell-depleted mice (Table 1, boxes). The percentage of hybridomas producing neutralizing antibodies remained consistently high at 12–16% of all hybridomas in fusions from spleen cells of CD8⁺ T-cell-depleted mice. But in normal mice the percentage of

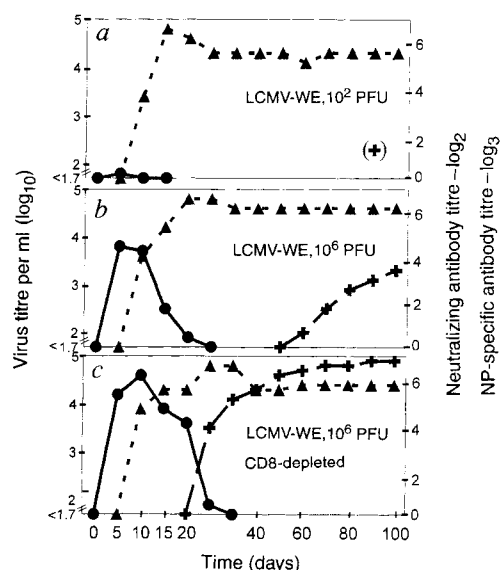


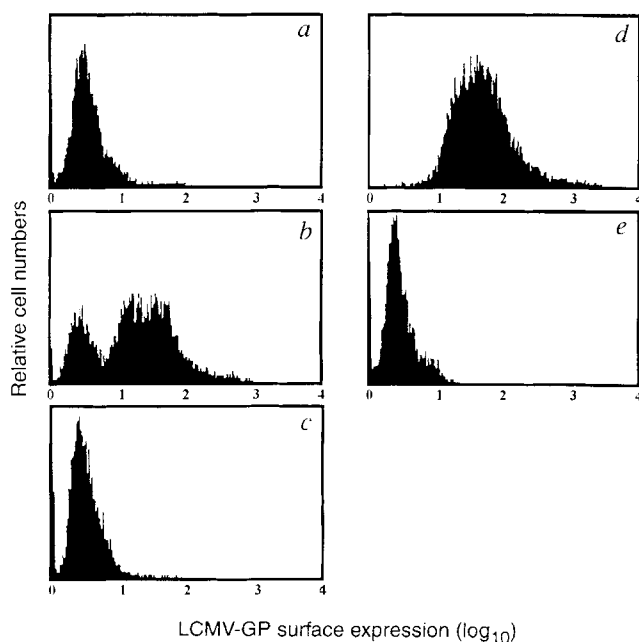
FIG. 1 Kinetics of neutralizing and non-neutralizing antibody responses to LCMV. a, BALB/c mice were infected i.v. with 10² PFU of LCMV-WE. Virus titre (●) in the blood of these mice is usually not detectable. Nucleoprotein-specific antibodies (▲) appear between days 6 and 8 after infection and stay at a constant level during the whole observation period; the cross in parentheses indicates that only 20% of BALB/c mice infected with 10² PFU LCMV-WE developed measurable neutralizing antibodies within 100 d of infection. b, BALB/c mice infected i.v. with 10⁶ PFU of LCMV-WE eliminated virus (●) from the blood by days 15–25 after infection. The nucleoprotein-specific antibody titre (▲) was comparable to that after low-dose (10² PFU) LCMV infection. Neutralizing antibodies (crosses) were found by around day 60 and later after infection. c, BALB/c mice were treated with rat anti-CD8 specific mAb (YTS 169.4) 3 and 1 day before LCMV-WE infection¹⁹. Mice were infected i.v. with 10⁶ PFU. LCMV titres (●) in the blood were increased compared with infected but otherwise untreated mice (see b). Kinetics of nucleoprotein-specific antibodies (▲) were comparable to those in infected mice from the other groups (a and b). Neutralizing antibodies (crosses) were found between days 20 and 25 after infection. Similar results were obtained in CD8-depleted mice after low-dose LCMV infection. All experiments shown used BALB/c mice, but similar results were obtained with C57BL/6 mice (not shown). All values for viral titres (●) are means of values from 6–8 individual mice; s.e.m. values of viral titres were all <0.40; s.e.m.s for neutralizing antibody titres were <0.66 and NP-ELISA titres were <0.15.

METHODS. Anti-LCMV-NP-titres were measured by ELISA and neutralizing antibody titres by a neutralization assay as described⁶. The neutralizing titre was measured as $-\log_2$, starting with a 1:20 dilution of serum and was defined as the dilution causing half-maximal reduction of plaques of LCMV with the same amount of virus incubated with control serum. NP-ELISA titres were measured as $-\log_2$, starting with a 1:40 dilution, and were defined by the dilution causing half-maximal colour reaction.

hybridomas producing neutralizing antibodies dropped markedly after day 4, and only one such hybridoma was isolated between days 10 and 25 after infection (Table 1, boxes).

Unexpectedly, we found that many hybridoma supernatants contained infectious LCMV. Further analysis showed that only those hybridoma supernatants that contained neutralizing antibodies were LCMV-positive. To eliminate infectious LCMV, supernatants were inactivated with ultraviolet light or treated with glycine-HCl buffer⁵, pH 2.8, when they were found to have potent neutralizing activity. In contrast, almost no supernatants with NP-specific antibodies contained infectious virus. As shown in Table 1, almost all (51/59) of the neutralizing-antibody-producing hybridomas, but very few (2/107) of the NP-specific hybridomas, were infected with LCMV.

Analysis of LCMV-infected virus-neutralizing antibody-secreting hybridomas showed that some expressed the LCMV-GP on the cell surface homogeneously, whereas others gave heterogeneous



staining (Fig. 2). After 3 subclonings, at 0.3 cells per well without feeder cells to avoid reinfection, all LCMV-infected clones produced neutralizing antibodies, whereas uninfected clones either did not secrete antibodies or secreted antibodies that did not neutralize LCMV (Fig. 2). It should have been possible⁵ to find LCMV-negative clones secreting neutralizing antibodies that had lost the virus during subcloning; but this was not found after 1–3 rounds of subcloning. The homogeneous hybridoma cultures remained stably infected for up to six months, that is, for as long as they were tested. However, by repetitive subclonings at 0.3 to 0.1 cells per well without feeder cells, rare hybridomas free of virus were isolated (Fig. 2). These virus-free subcloned produced 2–3 times more neutralizing activity than infected ones. Neutralizing activity, expressed as standard neutralizing titres of our monoclonal antibodies, were in the range of 1:2–1:8 neutralizing titres at $1 \mu\text{g ml}^{-1}$ (comparable to the standard neutralizing monoclonal antibody KL25 with a titre of 1:4.8 at $1 \mu\text{g ml}^{-1}$; ref. 16).

We then investigated whether the neutralizing-antibody-secreting cells were infected in the host or during fusion with the myeloma cells. The results showed that: (1) it was unlikely that LCMV infected hybridomas during the fusion process because LCMV-NP-specific hybridomas from the same fusion were not infected; (2) hybridomas specific for a variety of third-party antigens, including LCMV-NP or the myeloma cell line P3X63Ag8, could not be infected with LCMV, either *in vitro* or *in vivo* in LCMV carrier mice (results not shown); also, LCMV-neutralizing-antibody-producing hybridomas rendered virus-free by subcloning could not be infected (6 of 6 tested; see example in Fig. 3c), probably because of the general lack of membrane-anchored surface immunoglobulin and the resistance to LCMV infection seen for all hybridomas; (3) infected third-party-antigen- or NP-specific hybridomas were not obtained under conditions in which LCMV (5×10^8 PFU in $10 \mu\text{l}$) was added directly to the cell pellet before fusion (data not shown), so infection of B cells secreting LCMV-neutralizing antibodies must have occurred before fusion.

Taken together, our data indicate that LCMV-neutralizing, membrane-bound immunoglobulin on B cells serves as a specific virus receptor for infection of B cells with LCMV. Because LCMV-NP is not exposed on the virus surface, NP-specific B cells cannot take up infectious LCMV particles, but can only take up and process cell or virus fragments containing nucleoprotein.

Viral infection and the expression of major histocompatibility

FIG. 2 Expression of LCMV-specific antigen on neutralizing-antibody-producing hybridomas was tested by flow cytometric analysis. One example of 15 tested and subcloned hybridomas is shown; 9 of 15 gave a homogeneous staining pattern. The hybridoma shown is representative of 6 out of the 15 hybridomas that initially gave heterogeneous LCMV-GP staining. After one round of subcloning, we found homogeneously infected hybridomas producing neutralizing antibodies, whereas hybridoma cultures that stained negative for LCMV-GP did not produce neutralizing antibodies. a, Second-antibody control staining of an LCMV-infected hybridoma (4/4F7) with FITC-labelled goat anti-rabbit (IgG) antibody. b, Expression of LCMV-specific antigens on hybridoma cells (4/4F7) producing LCMV-specific neutralizing antibodies staining with a rabbit anti-LCMV hyperimmune serum, followed by an FITC-labelled goat anti-rabbit (IgG) antibody. After subcloning, hybridomas were re-tested for virus in the culture supernatant for expression of virus antigen on the surface of the clones and for secretion of neutralizing antibodies (c, d). The uninfected clone (c) did not produce LCMV-specific neutralizing antibodies. Neutralizing antibodies were found in the culture supernatant of the LCMV-infected clone (d) after the supernatant had been inactivated. e, Lack of LCMV antigen expression on a representative cloned hybridoma producing anti-LCMV-NP-specific antibodies.

METHODS. 4×10^5 hybridoma cells were incubated with a rabbit anti-LCMV-specific hyperimmune serum (1:10 dilution) for 45–60 min at 4°C and washed 3 times with PBS/0.05% EDTA, followed by incubation with FITC-labelled goat anti-rabbit antibody (TAGO; 1:50 dilution) for 30 min at 4°C . After 3 washes, cells were fixed with BSS/4% paraformaldehyde for flow cytometric analysis. Viable cells were gated by a combination of forward light scatter and 90° side scatter and were analysed on a FACSCAN (Becton Dickinson).

complex (MHC) class I molecules should render the specific LCMV-neutralizing B cells susceptible to lysis by specific cytotoxic T cells. Therefore, the LCMV-infected, neutralizing-antibody-producing hybridomas were tested as target cells for LCMV-specific cytotoxic T cells *in vitro*. We found that LCMV-infected

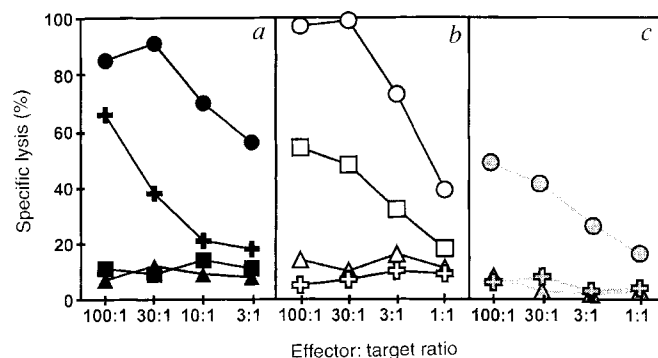


FIG. 3 LCMV infected hybridomas as target cells for LCMV-specific cytotoxic T cells. a, ^{51}Cr release assay using two LCMV-infected neutralizing antibody-secreting hybridomas (circles and crosses) and two uninfected NP-specific antibody-secreting hybridomas (squares and triangles) as target cells. Effector cells were spleen cells from a BALB/c mouse ($H-2^d$) seven days after infection with LCMV-WE 10^2 PFU. b, Uninfected NP-specific antibody-secreting hybridomas were either incubated for 48 h with LCMV, m.o.i. 0.01 (triangles), or labelled with LCMV-specific peptide NP 118–126 (squares). Also, MHC-matched fibroblasts were either incubated with the NP 118–126 peptide (circles) or were used without further treatment as controls (crosses). c, Hybridoma (9/4A5) producing LCMV-neutralizing antibody that was originally infected but was then rendered virus-free by subcloning, was incubated for 48 h with LCMV, m.o.i. 0.01, 1 or 100 (shaded crosses; data for 1 and 100 not shown), or labelled with LCMV-specific peptide NP 118–126 (circles), or used as target without any further treatment (triangles).

METHODS. Hybridomas used as target cells were incubated with $50 \mu\text{Ci}$ of ^{51}Cr per ml overnight, were washed extensively and incubated at different effector to target ratios with effector spleen cells. Peptide labelling of the hybridomas was done for 2 h before the assay at a peptide concentration of 10^{-6} M. Fibroblasts were labelled with $200 \mu\text{Ci}$ ^{51}Cr per ml and peptide NP 118–126 for 2 h before assaying. CTL activity was detected in a 6-h ^{51}Cr release assay. Spontaneous lysis was $<25\%$.

TABLE 1 Correlation of frequency and specificity of LCMV-specific hybridomas with CD8⁺ T-cell competence and time after LCMV infection

Treatment of host* (Days after infection)	Hybridomas per fusion in 960 wells* (total number) %		LCMV-infected hybridoma cultures (positive/total) %		Hybridomas secreting LCMV-specific antibodies† (positive/total) %				LCMV-producing hybridomas‡ (positive/total) %			
					Neutralizing	NP-binding	Neutralizing	NP-binding	Neutralizing	NP-binding	Neutralizing	NP-binding
d 2	(94)	10	(13/94)	14	(0/94)	0	(0/94)	0	(0/0)	—	(0/0)	—
d 2, anti-CD8	(56)	6	(6/56)	11	(0/56)	0	(0/56)	0	(0/0)	—	(0/0)	—
d 4	(46)	5	(10/46)	22	(4/46)	9§	(7/46)	15	(4/4)	100	(0/7)	0
d 4, anti-CD8	(85)	9	(31/85)	37	(13/85)	15	(10/85)	9	(11/13)	85	(1/10)	10
d 6	(94)	10	(11/94)	12	(3/94)	3	(18/94)	19	(3/3)	100	(0/18)	0
d 6, anti-CD8	(104)	11	(21/104)	20	(17/104)	16	(20/104)	19	(17/17)¶	100	(1/20)	5
d 10	(42)	4	(3/42)	7	(0/42)	0	(8/42)	19	(0/0)	—	(0/8)	0
d 10, anti-CD8	(34)	4	(5/34)	15	(5/34)	15	(6/34)	18	(5/5)¶	100	(0/6)	0
d 25	(47)	5	(3/47)	7	(1/47)	2	(13/47)	28	(0/1)	0	(0/13)	0
d 25, anti-CD8	(128)	13¶	(18/128)	14	(16/128)	13	(18/128)	14	(11/16)	69	(0/18)	0

* Fusions of spleen cells were done on days 2, 4, 6, 10 and 25 after infection with 10⁶ PFU of LCMV. Data are representative of one fusion for each day, using established protocols. Fusions were repeated independently at least twice with comparable results. Antibody assays determined by ELISA and neutralization and anti-CD8 treatment of mice have been described⁶.

† All hybridomas on day 2 (d 2) and d 4 secreted IgM. However, at later times most of the hybridomas produced IgG; 57% on d 6, 74% on d 10 and 79% on d 20.

‡ LCMV isolates from infected hybridomas were not variants that had escaped neutralization as they were all still neutralized by standard and the newly isolated neutralizing antibodies described here.

§ Key data on percentages of hybridomas secreting neutralizing, versus those secreting non-neutralizing, antibodies in LCMV-infected normal or CD8⁺ T-cell-depleted mice are boxed.

¶ Some of the hybridomas were initially probably not clonal. During further culturing and subcloning, some hybridomas were lost. Representations of some ≥ 3 times subcloned, stable, homogeneous and independent hybridomas (from day 6, anti-CD8 fusion: 13/8D2 IgM, titre: 2.5 at 1 µg ml⁻¹; 13/1A6 IgG2a, titre: 1.8; 13/3H12 IgG1, titre: 3.0, 4/4F7 IgG2a, titre: 5.4. From day 10, anti-CD8 fusion: 7/15A9 IgGb, titre: 8.4; 9/4A5 IgG2a, titre: 4.9; standard LCMV-neutralizing antibody KL25: titre, 4.8) were studied in detail. Additional fusions using BALB/c and C57BL/6 mice yielding more than 2,000 primary hybridomas with about 10% cultures with anti-LCMV specificity confirmed these results.

¶¶ The relative increased number of hybridomas on day 25 in anti-CD8-treated mice reflects the known antigen persistence and continued stimulation at that time.

hybridomas were killed by specific CD8⁺ T cells, whereas hybridomas producing antibodies directed against LCMV-NP were not (Fig. 3a). The latter hybridomas preincubated with the immunodominant cytotoxic-T-cell peptide NP 188–126 for H-2^d (refs 17, 18) were efficiently lysed (Fig. 3b). These results with hybridomas and the evidence from kinetic studies of neutralizing anti-LCMV antibody titres in mice suggested that neutralization-specific B cells were eliminated by LCMV-specific CD8⁺ T effector cells *in vivo*.

It could be argued that the difference in kinetics between the early appearance of NP-specific antibodies (days 6–8) compared with the later (days 15–25) appearance of neutralizing antibodies in the serum of CD8⁺ T-cell-depleted mice (Fig. 1) is not fully compatible with the proposed mechanism. The delay in neutralizing-antibody kinetics, however, may be due to prolonged high titres of circulating virus caused by the absence of antiviral CD8⁺ T cells. Whether this virus exhaustively absorbs neutralizing antibodies as they appear in serum and renders them undetectable remains to be investigated: this might explain why neutralizing antibodies are detectable as soon as the virus titre drops in the blood of CD8⁺ T-cell-depleted LCMV-infected mice, when CD8⁺ T-cell responses start to recover 8–10 days after depletion¹⁹.

It is possible that CD8⁺ or CD4⁺ T cells may destroy B cells that have taken up and presented antigen in association with class I or II MHC molecules^{20–22}. For example, B cells transformed by Epstein–Barr virus (EBV) and exposed to non-infectious HBV surface (HBs) antigen are susceptible to lysis by HBs-specific HLA class I- and possibly class II-restricted CTLs *in vitro*^{10,23}. It is important that much of the earlier evidence with non-infectious protein antigens and the results described here with LCMV-NP suggest that this mechanism does not play a significant role in the regulation of antibody responses to non-infectious antigens *in vivo*^{22,24,25}. Non-infectious antigens (including LCMV-NP) can induce CD8⁺ T-cell responses *in vivo* or *in vitro*²⁶, but these conditions are not able to downregulate specific B-cell responses to the same antigens *in vivo*. This indicates that B cells must be

infected to become targets for 'suppressive' cytotoxic T cells and that this does not occur if non-infectious forms of antigens (even of the highly expressed LCMV-NP⁶) are taken up by specific B cells *in vivo*.

Our results suggest that during LCMV infection, B cells specific for the viral surface antigens can be selectively and productively infected by LCMV through their membrane-anchored neutralizing-antibody receptor and are subsequently eliminated by virus-specific CTLs. In contrast, neutralizing-antibody-secreting hybridomas (and probably plasma cells) cannot any longer be infected because they lack the membrane-anchored surface immunoglobulin. It will be interesting to discover how and why B cells are able to support replication of non-cytopathic viruses, and how B cells resist infection by cytopathic viruses, as the latter induce early neutralizing antibodies that are essential for host survival^{27,28}.

An implication of our findings is that this mechanism might play a role in EBV infections²⁹ and they could explain observations in HBV² and HIV infections in humans, where non-neutralizing antibody responses also appear early and where the virus-specific neutralizing-antibody response has also been found to be strongly delayed. A highly specialized form of adaptation of host and virus may exist, in which some viruses 'use' immunological effector pathways to prevent early generation of protective neutralizing antibody responses, thereby permitting virus either to persist for a prolonged period or to establish a carrier state. □

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Deoxycytidyl transferase activity of yeast *REV1* protein

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MUTAGENESIS induced by DNA damage in *Saccharomyces cerevisiae* requires the products of the *REV1*, *REV3* and *REV7* genes¹. The Rev3 and Rev7 proteins are subunits of DNA polymerase- ζ ² (Pol- ζ), an enzyme whose sole function appears to be translesion synthesis³. Rev1 protein has weak homology with UmuC protein⁴, which facilitates translesion synthesis in *Escherichia coli* by an unknown mechanism. We show here that Rev1 protein has a deoxycytidyl transferase activity which transfers a dCMP residue from dCTP to the 3' end of a DNA primer in a template-dependent reaction. Efficient transfer occurred opposite a template abasic site, but ~20% transfer also occurred opposite a template guanine and ~10% opposite adenine or uracil; $\leq 1\%$ was seen opposite thymine or cytosine. Insertion of cytosine opposite an abasic site produced a terminus that was extended efficiently by Pol- ζ , but not by yeast Pol- α .

The deoxycytidyl transferase activity was discovered when testing the ability of Rev1 protein to enhance bypass of a template lesion by DNA polymerase ζ , using a glutathione-S-transferase (GST)-Rev1 fusion overexpressed in yeast and purified to near-homogeneity by affinity chromatography on glutathione-Sepharose (Fig. 1). The transferase activity was highly specific for dCTP and transferred a dCMP residue to the 3' end of a DNA primer in a template-dependent reaction. Transferase activity was proportional to enzyme concentration and the kinetics were linear up to at least 10 min, or until ~75% of the primers had been extended. Primers were not extended in the absence of a template, and the enzyme could not use ribonucleoside triphosphates. With the primer-template oligonucleotide pairs shown (Fig. 2), no primer extension was observed when the first template nucleotide was a T or a C, but two C residues were added to the primer paired with a template containing two adjacent G residues. These C residues

were added in a processive reaction as almost all of the extended primers contained two C residues, even under conditions where only a small fraction of the primer molecules were extended. A single C residue was also added opposite a template A but not a second residue, even though the template oligonucleotide contained two adjacent A residues. A single C residue was also added to oligo(dT)₂₅ poly(dA).

Previous experiments *in vivo*, demonstrating a marked preference for dCMP insertion opposite an abasic lesion in yeast⁵, prompted us to test whether the Rev1 transferase could insert a dCMP residue opposite this lesion. We constructed a template oligonucleotide with a specific dUMP residue and then treated the DNA with uracil-N-glycosylase to convert it to an abasic site. The transferase inserted dCMP opposite this abasic site about fivefold more efficiently than it did opposite a template G. Surprisingly, although the enzyme is unable to use a template T, dCMP was inserted with low efficiency opposite uracil, used in a control template. This was not a consequence of Rev1 containing uracil-N-glycosylase activity, because treatment of the template with Rev1 did not result in the alkali cleavability characteristic of an abasic site. Normalized to insertion opposite the abasic site, insertion efficiency opposite G was 20%, opposite A and U was 10%, and opposite C and T was 1% or less. Interestingly, only a single dCMP residue was added to the primer when the template contained two adjacent abasic sites, and the same true was when the abasic site was followed by guanine. In contrast, two dCMP nucleotides were added when a template uracil was followed by guanine.

The efficiency of insertion opposite a template abasic site suggests that Rev1 activity may assist in the first step in the bypass of these lesions, the insertion of a nucleotide opposite the lesion. We therefore examined the ability of Pol- ζ and yeast Pol- α to complete the bypass replication by extending the resulting 3' terminus (Fig. 3). In the absence of Rev1, Pol- ζ was capable of both inserting a nucleotide opposite the abasic site and further

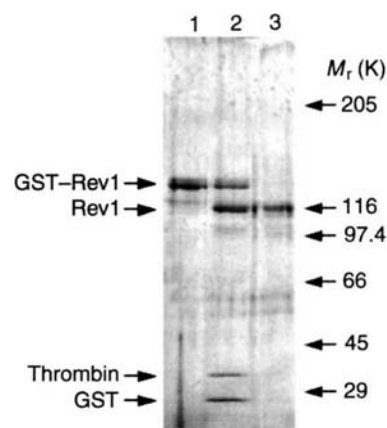
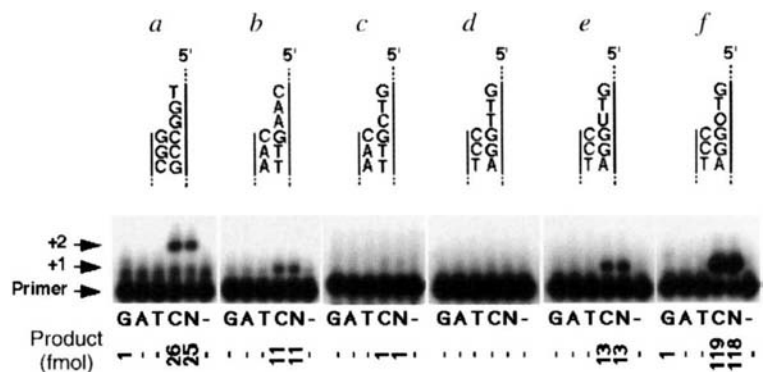


FIG. 1 SDS-polyacrylamide gel electrophoresis analysis of purified Rev1 proteins. Lane 1, eluate from the glutathione-Sepharose column, showing the ~140K GST-Rev1 fusion protein. Lane 2, products from cleavage at the thrombin-sensitive site at the GST-Rev1 fusion junction. Lane 3, the Rev1 cleavage product further purified by fractionation on a glycerol gradient.

METHODS. A GST-Rev1 fusion protein was overexpressed in yeast containing the plasmid pGST-REV1, constructed by inserting a 3.5-kb DNA fragment containing REV1 between the *Bam*HI and *Pst*I cloning sites of pGST, and purified by affinity chromatography on glutathione-Sepharose as described for the purification of a GST-Rev3 protein². About 300 μ g GST-Rev1 was obtained from 5 g of yeast paste. A portion (14 μ g) of the glutathione-Sepharose fraction was incubated for 15 h at 4°C with 3 μ g of thrombin and sedimented on a 10–30% glycerol gradient containing 0.5 M NaCl. Electrophoresis was on a 5–15% polyacrylamide gel and the gel was stained with silver.

FIG. 2 Rev1 protein is a deoxycytidyl transferase. Primer extension assays, using no dNTP (–), a single dNTP (G,A,T,C), or all four dNTPs (N), and templates with different nucleotides adjacent to the primer terminus. O, abasic site. +1 (+2), primer extended by one (two) nucleotide(s).

METHODS. Transferase reactions (10 μ l) contained 25 mM potassium phosphate (pH 7.4), 6 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg ml⁻¹ acetylated BSA, 10% glycerol, 20 nM 5' ³²P-labelled oligonucleotide primer annealed to an oligonucleotide template, 100 μ M dNTP as indicated, and 5 ng GST-Rev1. After incubation at 30 °C for 2.5 min, reactions were terminated with 20 μ l of 20 mM EDTA, 95% formamide, and the reaction products were resolved on a 12% denaturing polyacrylamide gel and visualized by autoradiography. The fraction of primers extended was determined by phosphorimaging and used to calculate the amount (in fmol) of product formed in the 10- μ l reaction. Dash indicates that no product (< 0.5 fmol) was detected. Labelled primers were: a, 5'-ACGACGTTGTAAACGACGG-3'; b, 5'-GACGGCCAGTGAATTCTCCAC-3'; c, 5'-ACGACGTTGTAAAC-3'; d, e, f, 5'-GACGGCCAGTGAATTCTCC-3'. Templates were: a–d, 5'-ACAGGAAACAGCTATGACCATGATTCAGTGGCAAGTTGGAGAATTCAGTGGCCGTCGTTTTACAACGTCGT-3'; e, 5'-CAGTGGCAAGTUGGAGAATTCAGTGGCCGTC-3'; for f, the template used in e was treated with *E. coli* uracil DNA-glycosylase to remove the single uracil and create an



abasic site. Control reactions with Klenow fragment, which is blocked by an abasic site, indicated that <1% of the treated templates retained a uracil residue, and sizing of the template in f, 5'-labelled with ³²P and treated with uracil-N-glycosylase, by 20% denaturing PAGE indicated that $\geq 95\%$ of templates were intact.

extending the primer, but with only 7% the efficiency seen on the uracil-containing control template (product, abasic template/uracil template = 2/27). With the addition of Rev1, bypass efficiency rose to 30–40% (product, abasic template/uracil template = 9/19 or 9/27), however, a result similar to that observed *in vivo*⁵. No detectable bypass was seen with Pol- α , whether Rev1 was present or not. These experiments were carried out with limiting polymerase to compare accurately the efficiency of synthesis on damaged and undamaged templates. As only a fraction of the primers were extended, the products resulted largely from a single polymerase–primer/template binding event. With a template uracil adjacent to the primer terminus, both DNA polymerases were capable of extending the primer, independent of the presence of Rev1, although Rev1 slightly reduced the activity of Pol- ζ and stimulated the activity of Pol- α . As we added sufficient Rev1 to these reactions to extend $\sim 75\%$ of the primers, it is likely that the enhanced bypass by Pol- ζ results from addition of a dCMP opposite the abasic site. However, we cannot rule out the possibility that Rev1 stimulates Pol- ζ bypass by some other mechanism.

A Rev1–MalE fusion protein (but not Rev7–MalE, used as a control), expressed in *E. coli* and purified by affinity chromato-

graphy on amylose resin, also has deoxycytidyl transferase activity, indicating that the activity is associated with the yeast Rev1 itself and does not require either the glutathione-S-transferase moiety or some associated contaminating yeast protein (Fig. 4a). Similarly, transferase activity confractionated with 8S GST–Rev1 following sedimentation through a glycerol gradient and, more particularly, with 5.5S Rev1 alone after cleavage of the thrombin-sensitive site at the junction of the GST–Rev1 fusion (Fig. 4b). The specific activities of GST–Rev1, thrombin-cleaved GST–Rev1, and the peak fractions of both glycerol gradients were very similar, indicating that the GST moiety has no effect on transferase activity.

As Rev1 efficiently inserts dCMP opposite an abasic site *in vitro* and preferential insertion of dCMP opposite this lesion is also seen *in vivo*⁵, it is likely that the transferase functions in the bypass of abasic sites. If a significant fraction of abasic sites generated in the cell come from the loss of guanine, insertion of dCMP could be a strategy to reduce mutations. The yeast genome is likely to suffer only 1–2 spontaneous depurinations per division cycle⁶, and only about 60% of these depurinations are believed to result from the loss of guanine. Furthermore, most of these are expected to be repaired efficiently. Rev1 dCMP transferase activity may there-

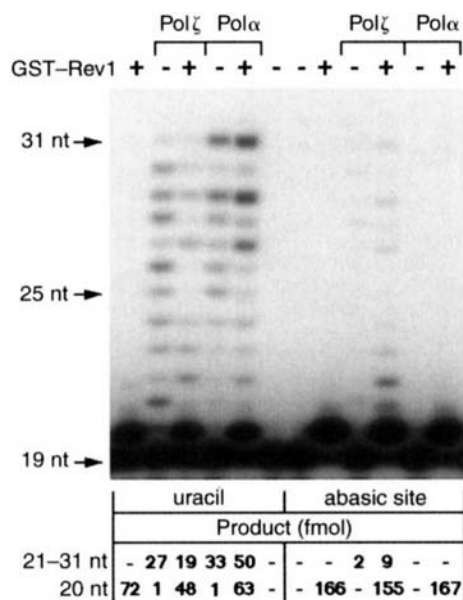


FIG. 3 DNA synthesis past a template abasic site using GST–Rev1 together with either yeast Pol- ζ or yeast Pol- α . Left lanes, control template with uracil adjacent to primer terminus; right lanes, templates with an abasic site at this position. nt, Nucleotides.

METHODS. Reactions were carried out using each dNTP at 100 μ M as for Fig. 2, using template e (containing uracil; Fig. 2) and template f (containing an abasic site, O; Fig. 2). Reactions contained, as indicated: 14 ng GST–Rev1, 6 ng yeast Pol- ζ (GST–Rev3: Rev7)², and 0.2 ng yeast Pol- α .

FIG. 4. Copurification of deoxycytidyl transferase activity and Rev1. *a*, A MalE–Rev1 fusion protein also has deoxycytidyl transferase activity. Primer extension assays with primer/templates *a*, *e* and *f* (Fig. 2) were done using MalE–Rev1 and with primer/template *a* using a preparation of a MalE–Rev7 fusion protein as a control. *b*, Copurification of deoxycytidyl transferase activity and Rev1 during glycerol gradient sedimentation. Primer extension assay (top) and SDS–PAGE analysis of protein (bottom), either before cleavage of the GST–Rev1 fusion protein by thrombin, or after such cleavage. Lanes L, sample loaded on gradient; other lanes contain material from the numbered fractions indicated.

METHODS. *a*, MalE (a maltose-binding protein) fusion proteins were expressed using the vector pMAL-c2 and purified using a kit from New England Biolabs according to the manufacturer's instructions. Transferase reactions are described in Fig. 2 legend, using all four nucleotides and 250 ng MalE–Rev7 (control) or 3 ng full-length MalE–Rev1, containing ~80 ng total protein. About 95% of the MalE–Rev1 fusion protein molecules obtained with this expression system were smaller than the expected 152K. *b*, GST–Rev1 (7 µg) with or without thrombin (1.5 µg) were incubated as described for Fig. 1, and sedimented separately through linear 10–30% glycerol gradients for 25 h at 50,000 r.p.m. in an SW-60 rotor. Fractions (170 µl) were collected from the bottom of tubes and 16-µl samples analysed by SDS–PAGE and silver staining. Transferase reactions were carried out as for

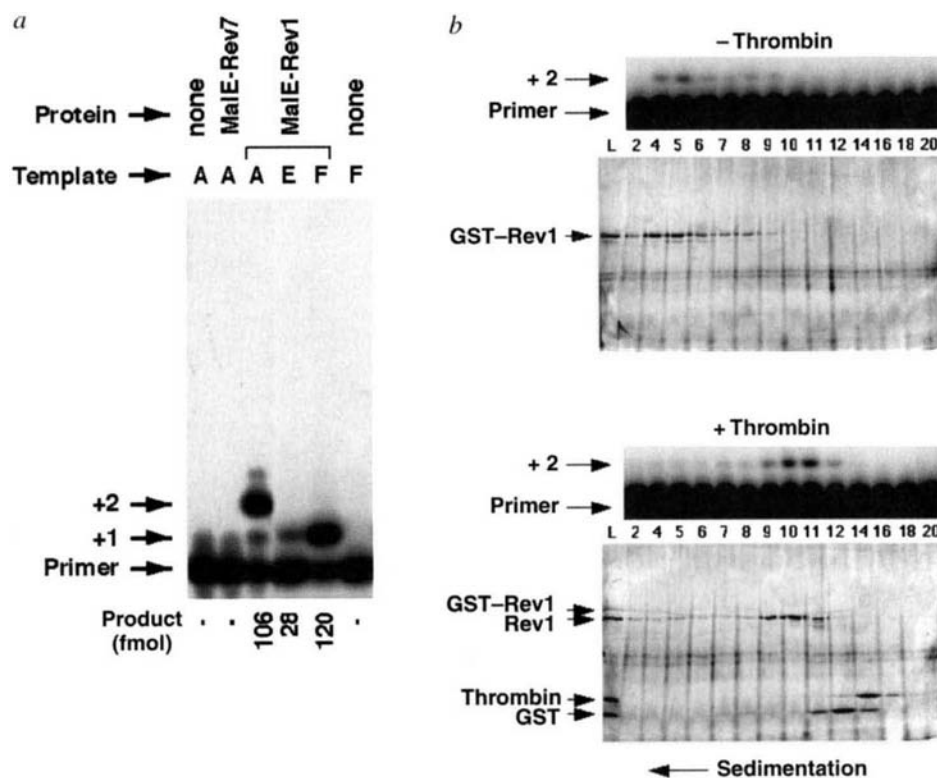


Fig. 2 with primer/template *a* and 1.5 µl of a 1:20 dilution of the indicated fractions. About 60% of the loaded proteins were recovered in peak fractions.

fore also be concerned with abasic sites resulting from the enzymatic removal of damaged guanine, because this nucleotide is particularly susceptible to oxidative damage, although if base-excision repair is a concerted process the abasic intermediate may be short-lived. The transferase may also occasionally be concerned directly with the bypass of oxidative damage, but a major role of this kind is unlikely as 8-oxo guanine, one of the most common lesions, rarely blocks normal replication, a precondition for Rev1 action. In addition to its role in the bypass of abasic sites, it is likely that Rev1 has another function in translesion synthesis. *REV1* function is required for ultraviolet mutagenesis in yeast, but dCMP is not inserted opposite ultraviolet photoproducts *in vivo*^{7–9}. In keeping with this, we find that Rev1 does not insert dCMP or any other nucleotide opposite a T–T dimer *in vitro* (data not shown). Finally, Rev1 seems to represent a new category of nucleotide-polymerizing enzyme. Unlike terminal nucleotidyl transferases¹⁰ or DNA polymerases, the Rev1 transferase use only dCTP, at least with the template primers tested, and the reaction has unusual template requirements. In addition, Rev1 shares no motifs or sequence homologies with either the terminal transferases or with any of the DNA polymerases. □

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Oxidative DNA damage through long-range electron transfer

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THE possibility has been considered for almost forty years that the DNA double helix, which contains a π -stacked array of heterocyclic base pairs, could be a suitable medium for the migration of charge over long molecular distances^{1–11}. This notion of high charge mobility is a critical consideration with respect to DNA damage. We have previously found^{7–10} that the DNA double helix can serve as a molecular bridge for photo-induced electron transfer between metallointercalators, with fast rates ($\geq 10^{10} \text{ s}^{-1}$)¹⁰ and with quenching over a long distance ($> 40 \text{ Å}$)⁸. Here we use a metallointercalator to introduce a photoexcited hole into the DNA π -stack at a specific site in order to evaluate oxidative damage to DNA from a distance. Oligomeric DNA duplexes were prepared with a rhodium intercalator covalently attached to one end and separated spatially from 5'-GG-3' doublet sites of oxidation. Rhodium-induced photo-oxidation occurs specifically at the 5'-G in the 5'-GG-3' doublets and is observed up to 37 Å away from the site of rhodium intercalation. We find that the yield of oxidative damage depends sensitively upon oxidation potential and π -stacking, but not on distance. These results demonstrate directly that oxidative damage to DNA may be promoted from a remote site as a result of hole migration through the DNA π -stack.

Both experimental studies comparing reactivities of photo-

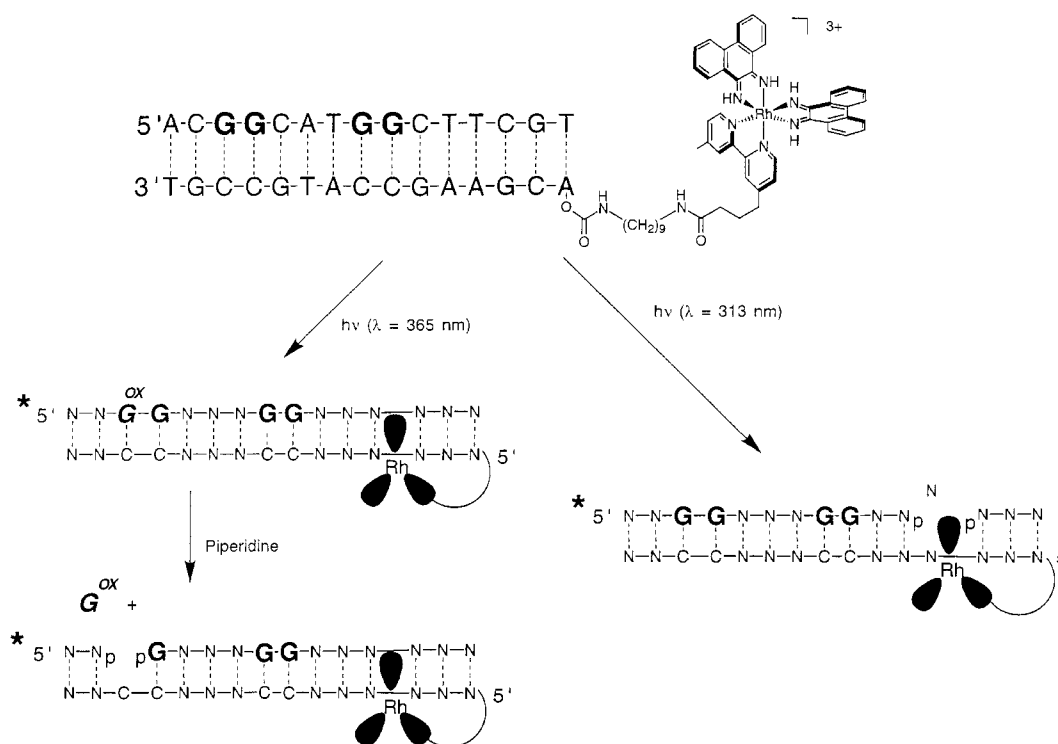


FIG. 1 A DNA duplex containing 5'-GG-3' doublet oxidation sites, modified by covalent attachment of the functionalized rhodium intercalator, and its wavelength-dependent pathways for photoreaction. Irradiation at low energy (365 nm) leads to photoactivated electron transfer from guanine to the photoexcited tethered rhodium complex. The site of guanine oxidation (G^{ox}) is revealed following treatment of the modified oligomer with piperidine, which leads to release of the modified base and strand cleavage¹⁸ to produce 3'- and 5'-phosphate termini on either side of the lesion, as shown for one of the two 5'-GG-3' doublets. Alternatively, irradiation at higher energy (313 nm) leads to direct strand cleavage through C3'-hydrogen abstraction by the rhodium complex intercalated at the site. Products of direct DNA photocleavage by phi complexes of rhodium include free bases and 3'- and 5'-phosphate termini²¹. This DNA cleavage chemistry, which can occur equally at all nucleotide positions, is used to mark sites of binding by the rhodium complex on the DNA helix. Only one of the four possible sites of strand cleavage is shown.

METHODS. Rhodium-modified oligonucleotide conjugates were prepared as follows: in order to activate $Rh(phi)_2bpy^{3+}$ (where bpy is 4-butyric acid 4'-methyl bipyridine) as the *N*-succinimidyl ester, the metal complex

(10 μ mol), together with *N,N,N',N'*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU; 20 μ mol) and *N,N*-diisopropylethylamine (DIEA; 20 μ mol) were dissolved in 0.5 ml of 1:1:1 $CH_3CN/CH_2Cl_2/CH_3OH$ and the solution stirred for 1 h at ambient temperature. Following activation, excess DIEA (100 μ mol) was added, and the solution combined with controlled-pore glass beads (2,000 Å pore; 1 μ mol loading) functionalized with the appropriate oligonucleotide (standard phosphoramidite synthesis) appended with a nonamethylene amino-alkyl linker³⁰. The resulting slurry was stirred gently for 12 h at ambient temperature to effect coupling, after which beads were isolated by filtration and washed extensively with CH_3OH and CH_2Cl_2 . To liberate the conjugate from the solid support, the beads were added to 0.75 ml aqueous ammonia (30%) and heated at 60 °C for 6 h. After filtration, the crude mixture was purified by reverse-phase HPLC (C_4 column support; ammonium acetate/ CH_3CN eluant). Coupling of racemic $Rh(phi)_2bpy^{3+}$ leads to the formation of Δ and Λ diastereomic conjugates which can be separated by HPLC. Characterization was by UV-visible spectroscopy, enzymatic digestion and base analysis, mass spectrometry and non-denaturing electrophoresis.

oxidants with different duplex sequences and calculations have indicated that the guanine base is the most easily oxidized^{12,13}. Guanine is also the major target of oxidative nucleic acid damage within the cell¹⁴. Different DNA-binding agents, including riboflavin^{15,16}, modified anthraquinones¹⁷, and naphthalimides¹² induce oxidative DNA damage specifically at the 5'-G of 5'-GG-3' doublets by electron transfer from guanine to the photoexcited acceptor. It is also found that the 5'-G with increasing electron-donating ability follows the series 5'-GT-3', 5'-GC-3' $\ll$ 5'-GA-3' < 5'-GG-3' < 5'-GGG-3' (ref. 12). We find that irradiation of $Rh(phi)_2DMB^{3+}$ (where phi is phenanthrenequinone diimine, and DMB is 4,4'-dimethylbipyridine) at 365 nm in the presence of duplex DNA also promotes damage specifically at the 5'-G of 5'-GG-3' doublets, as revealed¹⁸ through alkali-induced cleavage at these modified positions. Phi complexes of rhodium(III) bind DNA avidly ($K \geq 10^6 M^{-1}$) by intercalation in the major groove¹⁹⁻²¹, serve as potent photo-oxidants upon irradiation at low energies (365 nm)²², and promote direct strand cleavage²¹, marking sites of DNA binding, with photo-activation at higher energy (313 nm).

To introduce a photoexcited hole into the DNA site-specifically from a remote position, we have constructed a modified DNA

duplex in which the site of intercalation by the rhodium complex is separated spatially from preferred sites of DNA oxidation. As shown in Fig. 1, photocleavage of the rhodium-modified oligomer at 313 nm marks the site of intercalation by the rhodium complex, whereas photolysis at 365 nm followed by treatment with piperidine reveals the sites of oxidative DNA damage.

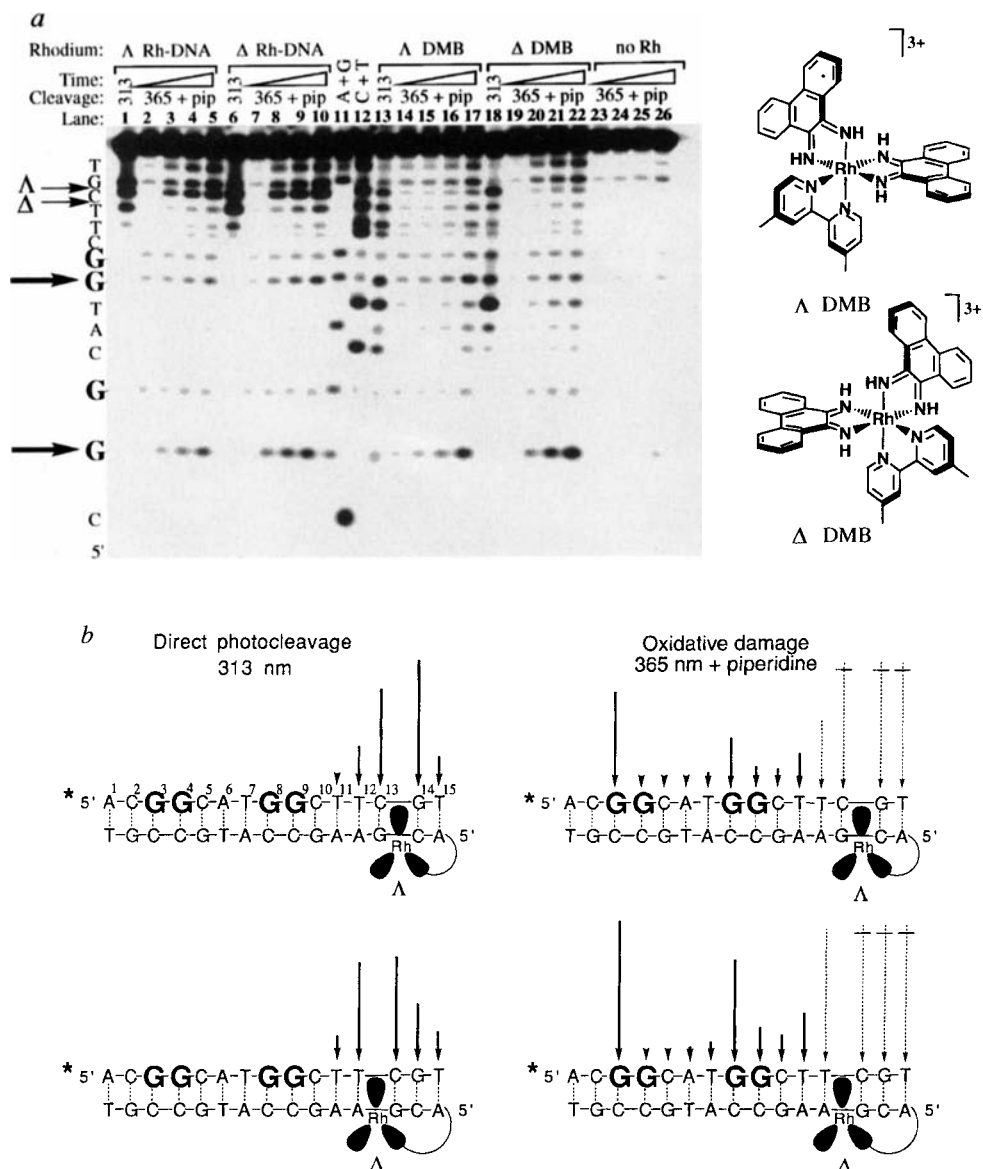
The results of photocleavage of a 5' ³²P-end-labelled oligonucleotide duplex containing two 5'-GG-3' doublets, either with covalently tethered rhodium or in the presence of non-covalently bound $Rh(phi)_2DMB^{3+}$, are shown in Fig. 2. The cleavage results observed upon irradiation of the covalently modified duplex at 313 nm clearly show that the covalently bound rhodium intercalator is confined to the tethered end of the duplex and is bound intramolecularly. In contrast, when bound non-covalently, both enantiomers of $Rh(phi)_2DMB^{3+}$ promote cleavage across the duplex, with particularly strong cleavage in the centre of the oligomer.

The sites of binding do not, however, reflect the sites of base oxidation. Rhodium-induced damage at 5'-GG-3' sites is evident with both covalently and non-covalently bound metal complexes and increases with increasing irradiation times at 365 nm. Also evident is damage (due to direct photochemistry) next to the intercalation site. For both Λ - and Δ - $Rh(phi)_2DMB^{3+}$, strong

FIG. 2 Comparison of oxidative damage and direct photocleavage on rhodium-modified DNA duplex diastereomers and on DNA duplexes with non-covalently bound $\text{Rh}(\text{phi})_2(\text{DMB})^{3+}$. The phosphorimager results on the 5' ^{32}P -end-labelled 15-mer after photolysis are shown in a; b summarizes quantitatively the sites (arrows) of direct photocleavage (left) and oxidative damage (right) for covalently modified rhodium-DNA conjugates (Δ top, Δ bottom), based upon the DNA photocleavage results in a. The 15-mer (Fig. 1) containing the 5'-GG-3' doublets with or without rhodium modification was 5' ^{32}P -end-labelled on the strand containing the 5'-GG-3' doublets. Lanes 1–5 contain the Δ diastereomer of covalently bound rhodium-DNA conjugate; lanes 6–10 contain the Δ diastereomer. Shown are the oligomers after photolysis at 313 nm for 8 min (lanes 1, 6) with direct photocleavage, or after photolysis at 365 nm for increasing irradiation times of 0 min (lanes 2, 7), 15 min (lanes 3, 8), 30 min (lanes 4, 9) or 60 min (lanes 5, 10), followed by treatment with piperidine. Cleavage was also examined on the unmodified duplex with Δ -Rh($\text{phi})_2\text{DMB}^{3+}$ (Δ DMB; lanes 13–17), Δ DMB (lanes 18–22), and in the absence of rhodium (lanes 23–26) either at 313 nm for 8 min (lanes 14, 19, 23), 15 min (lanes 15, 20, 24), 30 min (lanes 16, 21, 25) or 60 min (lanes 17, 22, 26) followed by treatment with piperidine. Lanes 11 and 12 contain the Maxam-Gilbert A + G and C + T sequencing reactions, respectively. Direct photocleavage of the Rh-DNA conjugates is confined to the tethered end of the duplex; the primary intercalation site for each enantiomer is marked by Δ or Δ . Because of the difference in patterns of direct photocleavage between covalently bound rhodium, which cleaves only near the tethered end of the oligomer, and non-covalently bound $\text{Rh}(\text{phi})_2\text{DMB}^{3+}$, which cleaves across the oligomer, the intercalation of the tethered rhodium complex must be confined to its own duplex. The sites of primary oxidative damage at the 5'-G within 5'-GG-3' doublets are indicated with arrows. On DNA restriction fragments, we find some variability in the yield of damage which appears to depend upon flanking sequence. The oxidative damage is also enhanced significantly in the presence of 100 μM Zn(II) and Cu(II) but not Mg(II). Irradiation was on 20- μl samples in 25 mM sodium cacodylate buffer, pH = 8, with [duplex] = [Rh] = 8 μM , using a 1,000 W Hg/Xe lamp with a mono-

chrometre. The 20% denaturing polyacrylamide gel was visualized by phosphorimager. The quantum yield for guanine damage is $\sim 1\%$ of the intrinsic photoreactivity of the rhodium complex at 365 nm ($\Phi_{\text{photoaquation}} = 5 \times 10^{-6}$), which is reasonable given the likelihood of non-productive back electron transfer. The direct photocleavage and oxidative damage are quantified in b for the Δ and Δ rhodium-DNA conjugates. For oxidative damage, 0 min irradiation intensities were subtracted from those with 60 min irradiation. Most damage next to the intercalation site following 365 nm irradiation does not require piperidine treatment for strand cleavage; these sites are indicated by dashed arrows.

reaction is observed at both 5'-GG-3' doublets, with greater intensity at the 5'-G. Significant cleavage is also evident next to T7, which probably results from direct cleavage at this most populated site. It is noteworthy that greater damage is apparent at G3 compared to G8. Importantly, with the rhodium intercalator covalently tethered, we also observe substantial base damage at the two 5'-GG-3' doublets, with cleavage primarily on the 5'-G. In addition, there is damage resulting from direct photocleavage next to the site of intercalation. Remarkably, the damage at the 5'-GG-3' doublets promoted by irradiation of the rhodium intercalator stacked near the end of the duplex requires long-range oxidation over distances of 17 Å and 34 Å for G8 and G3, respectively. The intensity of damage at the site distal to the tethered rhodium



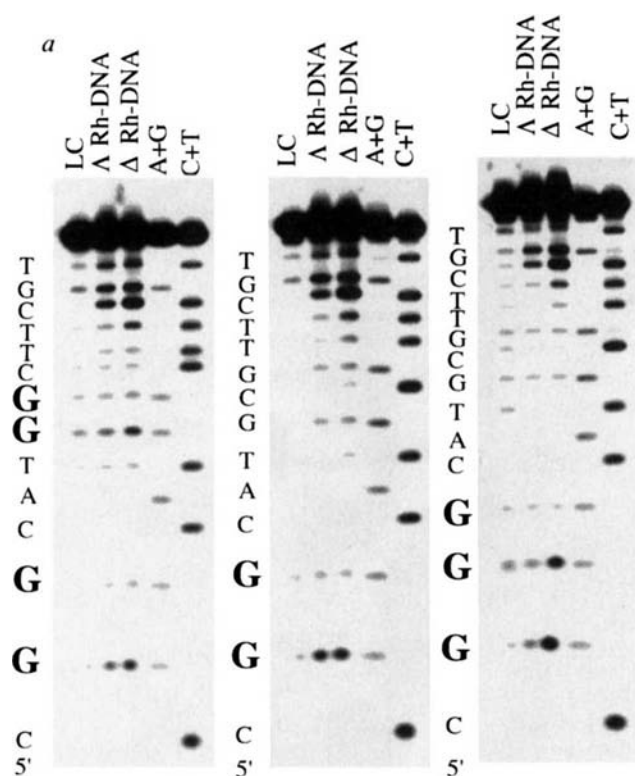


FIG. 3 Comparison of long-range oxidative damage on three covalently bound rhodium-modified DNA duplexes differing in sequence. *a*, Phosphorimaging of 20% denaturing polyacrylamide gels after photoreaction; *b*, summary of sites of photocleavage. In *a*, the oligomers examined are (from left to right in sets of 5 lanes) the 15-mer (Fig. 1) containing two 5'-GG-3' doublets, a 15-mer lacking the central 5'-GG-3' doublet, and a 16-mer containing a 5'-GGG-3' triplet. For each sequence the 5' 32 P-end-labelled oligomer is shown, either without rhodium modification but with irradiation (LC, light control), the Δ rhodium-DNA conjugate, the Δ rhodium-DNA conjugate, and Maxam-Gilbert sequencing reactions for A + G and C + T. Test samples were each irradiated for 1 h at 365 nm, followed by piperidine treatment and electrophoresis. In *b*, oxidative cleavage results from *a* for the

inconsistent with a diffusible species emanating from a site near the bound rhodium. The 5'-GG-3' site distal to the tethered rhodium is cleaved more than the central 5'-GG-3' site, and within each 5'-GG-3' doublet, it is the 5'-G distal to the rhodium that is preferentially damaged. In addition, singlet oxygen should react with all accessible guanines²³, and hydroxyl radicals should react with no site selectivity and without requiring piperidine treatment²⁴. Also, there is no significant enhancement in the presence of D₂O, as would be expected if $^1\text{O}_2$ were involved²³.

The major oxidation product after enzymatic digestion¹⁶ was found to be 8-oxo-2'-deoxyguanosine (8-oxo-dG), based upon its distinctive ultraviolet spectrum and its identical elution time after high-performance liquid chromatography (HPLC) with co-injected, authentic 8-oxo-dG. 8-oxo-dG represents the primary lesion in oxidative DNA damage²⁵. Its formation here requires dioxygen; there is no base damage under an argon atmosphere, and damage increases with increasing oxygen concentration. A possible role for dioxygen may be in trapping the guanine cation radical, with 8-oxo-dG as the final product. The photoinduced hole transfer nonetheless must occur within, at most, the microsecond timescale, based upon the lifetime of the rhodium excited state¹⁰.

To probe this long-range oxidative reaction on DNA, several additional rhodium-modified oligomers were prepared (Fig. 3). We compared photoreaction on the covalently modified 15-mer containing two 5'-GG-3' doublets to that on a modified 15-mer in which the central 5'-GGC-3' site was replaced by 5'-GCG-3' and to

Δ rhodium-DNA conjugates are shown. The solid arrows indicate sites of piperidine-dependent cleavage, whereas the cleavage marked by dashed arrows arises without treatment with alkali and therefore represents direct photocleavage. Quantification was done by subtraction of light control intensities. Specific activities for these three oligomers were equal as was the amount of radioactivity loaded per lane, enabling the intensity of cleavage among the different oligomers to be compared directly. Note that the 5'-GCG-3' segment of the middle 15-mer reveals slight damage, whereas no damage is evident at the identical segment of the 16-mer. This variation is understandable, given the high sensitivity of guanine residues to piperidine treatment.

that on a 16-mer in which the remaining 5'-GG-3' doublet was extended to a 5'-GGG-3' triplet. First, photoreaction on the 5'-G requires the 3'-G. Second, the presence of the central 5'-GG-3' doublet does not appear to diminish the intensity of photoreaction at the far 5'-GG-3' site. In fact, cleavage at the remote 5'-GG-3' doublet is slightly enhanced in the oligomer lacking the intervening 5'-GG-3' doublet. Thus, the central 5'-GG-3' doublet neither facilitates nor blocks long-range electron transfer through the duplex. Third, photoreaction at the 5'-GGG-3' triplet of the 16-mer is more intense than on the 5'-GG-3' doublet of the corresponding 15-mer. This result is consistent with earlier studies of relative electron-donating abilities of different guanine-containing sequences¹², although here such reaction requires long range electron transfer over a distance of 37 Å.

As seen in studies of DNA-mediated electron transfer between metallointercalators⁸⁻¹⁰, we also observe oxidative damage that is sensitive to stacking but appears to be insensitive to distance. For both the covalently and non-covalently bound rhodium, reaction of the Δ -isomer, which can stack more closely into the right-handed DNA duplex²⁶, is greater than that of the Λ -isomer. Moreover, comparison of cleavage intensities at the central and remote 5'-Gs indicates that over this range (17–37 Å), the yield of oxidized product is independent of the distance of separation from the oxidant. Comparison of the photoreaction at the central and remote 5'-GG-3' sites shows that these sites are separated on the DNA duplex by half a turn of helix, indicating that the intensity of photo-oxidation may also be relatively insensitive to whether

the rhodium intercalator and guanine base are positioned in phase or out of phase along the helix.

Although the reaction we have described involves long-range photoinduced electron transfer, the precise mechanism for this DNA-mediated charge transfer is not yet known. The oxidation of guanine could occur synchronously with reduction of the excited metallointercalator, that is, as a concerted, long-range event. Alternatively, the photoexcited metal complex could oxidize a base near the intercalation site, with subsequent migration of the resulting hole to its most thermodynamically favoured site, the 5'-G of the 5'-GG-3' doublet. However, intervening hole traps might be expected to prevent the hole from migrating to a distant 5'-GG-3' doublet. The damage we find at a remote site in the presence of an intervening 5'-GG-3' doublet may reflect a fast equilibration of holes across the DNA π -stack. To determine whether this reaction involves a delocalized radical or a weakly distance-dependent tunnelling will require kinetic measurements on a fast timescale. It appears that the efficiency of oxidative damage at a site correlates more closely to the oxidation potential of that site rather than to the distance of separation of the site from the oxidant.

Last, our results bear upon cellular DNA damage. Many carcinogenic agents damage DNA by introducing holes or electrons into the DNA helix²⁷. Radiation biology experiments have investigated whether charge can migrate over long distances through the DNA helix^{2,3}. We have used a tethered metallointercalator to inject charge at a precise location and show that damage to DNA can be promoted from a remote site along the helix under physiological conditions. 8-oxo-dG, the product of this reaction, promotes the misincorporation of dA opposite the site of damage²⁸, and radicals, once coupled into the DNA π -stack, may therefore migrate over extended molecular distances to generate these 8-oxo-dG lesions. This mechanism for long-range damage to DNA depends sensitively, however, upon the intervening DNA π -stack. Hence, we now need to consider whether DNA sequences and structures, through perturbations in π -stacking, and/or through variations in oxidation potential, act as electron sinks²⁹ or buffers to protect gene-encoding DNA sequences from remote damage. □

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Topology and reorganization of a human TFIID–promoter complex

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TRANSCRIPTION factor TFIID is a multiprotein complex composed of a TATA-box-binding subunit, TBP, and several tightly associated factors (TAFs)^{1,2}. Human TFIID–promoter interactions are restricted to the TATA-box region on most core promoters^{3,4} but extend over a large promoter region downstream of the TATA box and the transcription start site on the Ad2ML promoter^{3–6}. TFIID downstream interactions are thought to be functionally relevant because they can be induced by transcriptional activators^{7–10}, which in some cases requires TFIIA^{9,10}, result in stabilization of the TFIID–promoter complex^{9,10}, and correlate with increased recruitment of the remaining general transcription factors^{8,10}. Here we examine the topological organization of human TFIID complexes bound to the Ad2ML promoter. Our data provide insight into the relative disposition of DNA and several TFIID subunits, as well as evidence for DNA wrapping by TFIID, and suggest a direct role of TFIIA in the stable positioning of promoter DNA relative to TAFs.

We analysed the molecular topology of human TFIID and TFIID/TFIIA complexes with the Ad2ML promoter using a photocrosslinking method and the thymidine derivative 5-[N-(p-azidobenzoyl)-3-aminoallyl]-deoxyuridine triphosphate (N₃RdUTP)^{11,12}. N₃RdUTP places a highly photoreactive substituent about 9 to 10 Å away from the 5' position of the pyrimidine ring and therefore probes space in relative proximity to the major groove of the DNA^{11,12}. Seven TFIID subunits, including TAF_{II}250, TAF_{II}135, TAF_{II}95, TAF_{II}55, TAF_{II}46, p31 and TBP were selectively crosslinked to an Ad2ML promoter probe carrying randomly distributed radiolabelled nucleotides and thymidine-to-N₃RdUMP substitutions over the full length (Fig. 1a). To determine the positions of individual TFIID subunits relative to the promoter DNA, we then used Ad2ML promoter probes carrying N₃RdUMP and an adjacent radioactive label at specific sequence positions.

Representative results are shown in Fig. 1b–d and are summarized in Fig. 1e. TBP was efficiently crosslinked to positions –44/–43 (Fig. 1b, lanes 3, 5) and –40/–39 (data not shown). Crystallographic data^{13–15} show that the TATA-bound TBP core domain is too distant to account for efficient crosslinking to these positions. This suggests that the long (over 140 residues) N-terminal domain of human TBP, which was not analysed in the TBP–DNA co-crystals^{13–15}, is located upstream of the TATA box. As specific TBP binding is mediated by minor groove interactions^{14–17}, we observe only weak crosslinking to the TATA sequence. No TBP crosslinking occurred to sequences downstream of the TATA-box region, further emphasizing specific complex formation. Upstream of the TATA sequence we detect strong crosslinking of TAF_{II}135, TAF_{II}55 and p31 (Fig. 1b, lane 5). TAF_{II}135 is also strongly crosslinked to position –19 downstream of the TATA box (Fig. 1c, lane 11), whereas only TAF_{II}95 shows crosslinking to position –9/–8 (Fig. 1d, lane 3). At positions –2 and +3 we observe weak photocrosslinking of multiple TFIID subunits, including TAF_{II}250, TAF_{II}135, TAF_{II}95, TAF_{II}55, TAF_{II}46, and p31 (Fig. 1d, lane 7, and data not shown).

This result is indicative of variable positioning of this promoter region within the TFIID nucleoprotein complex. Indeed, human TFIID interactions with Ad2ML promoter sequences downstream

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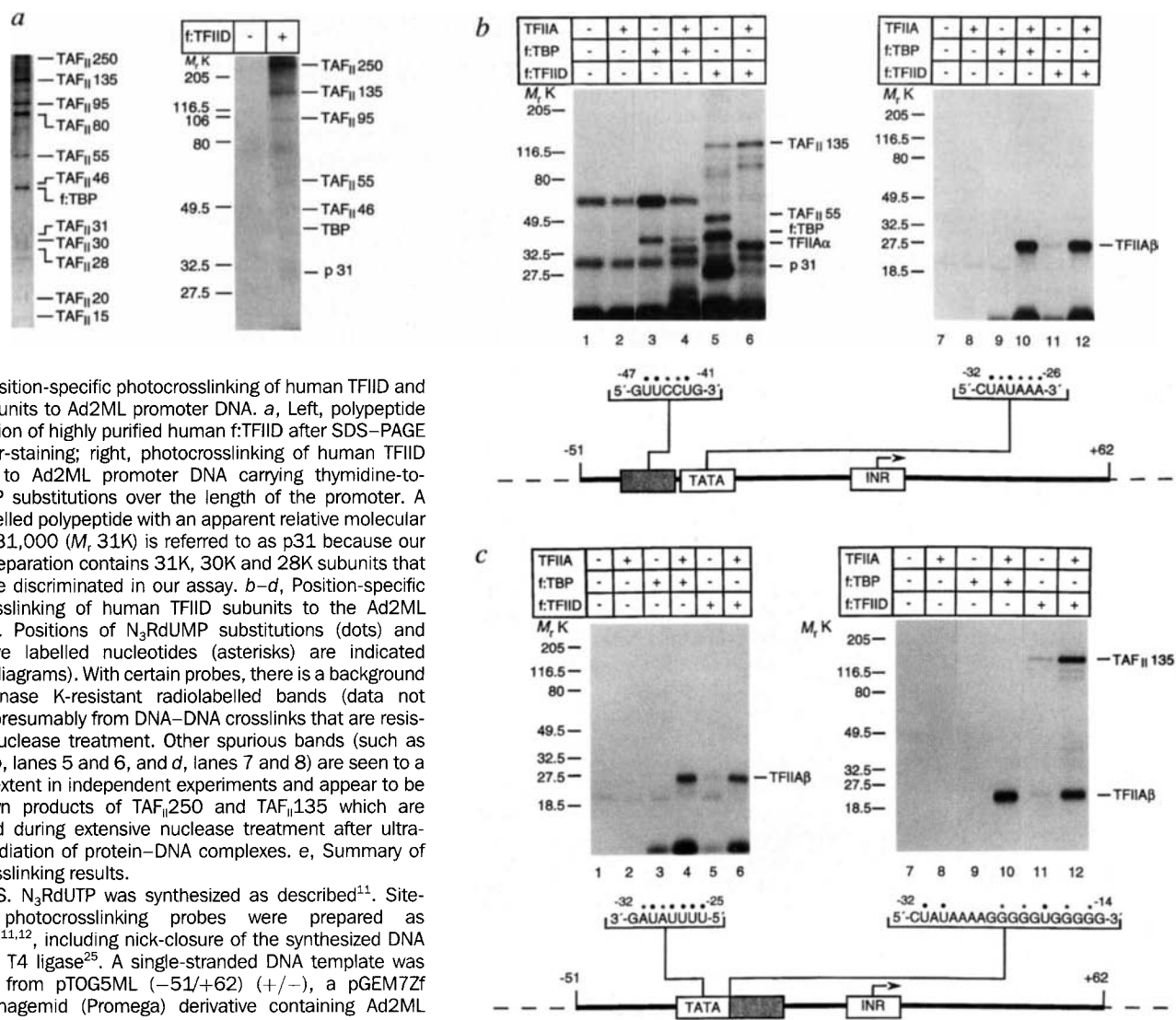


FIG. 1 Position-specific photocrosslinking of human TFIID and TFIIA subunits to Ad2ML promoter DNA. **a**, Left, polypeptide composition of highly purified human f:TFIID after SDS-PAGE and silver-staining; right, photocrosslinking of human TFIID subunits to Ad2ML promoter DNA carrying thymidine-to- N_3 RdUMP substitutions over the length of the promoter. A photolabelled polypeptide with an apparent relative molecular mass of 31,000 (M_r 31K) is referred to as p31 because our f:TFIID preparation contains 31K, 30K and 28K subunits that cannot be discriminated in our assay. **b-d**, Position-specific photocrosslinking of human TFIID subunits to the Ad2ML promoter. Positions of N_3 RdUMP substitutions (dots) and radioactive labelled nucleotides (asterisks) are indicated (bottom diagrams). With certain probes, there is a background of proteinase K-resistant radiolabelled bands (data not shown), presumably from DNA-DNA crosslinks that are resistant to nuclease treatment. Other spurious bands (such as those in **b**, lanes 5 and 6, and **d**, lanes 7 and 8) are seen to a variable extent in independent experiments and appear to be breakdown products of TAF_{II}250 and TAF_{II}135 which are generated during extensive nuclease treatment after ultra-violet irradiation of protein-DNA complexes. **e**, Summary of photocrosslinking results.

METHODS. N_3 RdUTP was synthesized as described¹¹. Site-specific photocrosslinking probes were prepared as described^{11,12}, including nick-closure of the synthesized DNA strand by T4 ligase²⁵. A single-stranded DNA template was prepared from pTOG5ML (-51/+62) (+/-), a pGEM7Zf (+/-) phagemid (Promega) derivative containing Ad2ML promoter sequences from -51 to +62 on a 268-bp *Apal/BamHI* insert. Complexes were formed as described for Fig. 2, using 5 fmol of 268-bp photocrosslinking probe, 0.5 footprinting units of either highly purified FLAG-tagged TFIID⁴ (f:TFIID) or recombinant f:TBP²⁶, and excess partially purified human TFIIA²⁷ containing no detectable DNA-binding activity (Fig. 2 and data not shown). Protein-DNA complexes were irradiated at 312 nm for 2 min, followed by nuclease

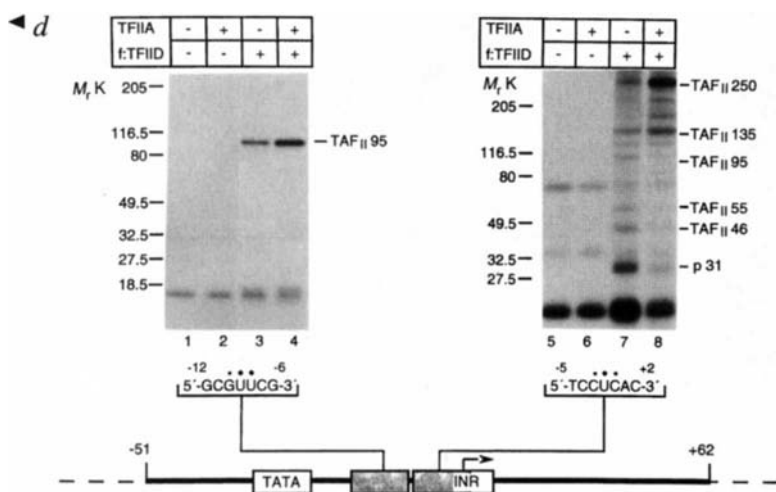
digestion with 1 μ g DNase I and 20 U micrococcal nuclease as described^{11,12}. Proteins were precipitated with trichloroacetic acid (TCA) and analysed by 10% SDS-PAGE or 6-20% gradient SDS-PAGE, followed by autoradiography.

of the TATA box region are largely sequence-independent³⁻⁵ and sensitive both to high salt concentrations and the presence of excess amounts of carrier DNA^{3,5} (T.O., unpublished observations; Fig. 2). One possible explanation is the apparent lack in human f:TFIID of a subunit homologous to *Drosophila* TAF_{II}150, which exhibits sequence-specific interactions downstream of the initiation site on certain promoters¹⁸.

Efficient photocrosslinking of TAFs was only observed at thymidine positions between -49 and +2, although human TFIID interactions on the Ad2ML promoter extend to +35 (refs 3-6). Although the photolabelling efficiency downstream of +3 was near the detection limit, we were able to detect very weak but reproducible crosslinking of TAF_{II}250 and TAF_{II}135 between +5 and +22 (data not shown). Thus, TFIID subunits appear not to be in close proximity to the major groove at thymidine positions downstream of the transcription start site. However, we cannot rule out the possibility that the bulky

N_3 RdUMP side chain interferes with efficient TFIID binding in this region.

We next investigated the possible effects of TFIIA on TFIID-DNA topology. Position-specific photocrosslinking of TFIIA subunits was observed with, and was absolutely dependent on, the presence of promoter-bound TBP or TFIID. For both TBP and TFIID, and in agreement with previously published results for TBP¹⁹, we localized the 21K TFIIA- β subunit at the TATA sequence opposite the TFIIB binding site and the 35K TFIIA- α subunit at promoter regions immediately upstream of the TATA box (Fig. 1b,c). Photocrosslinking of the 12K TFIIA- γ subunit was not detected. Strikingly, with TFIID as the promoter-binding factor, the binding of TFIIA- α upstream of the TATA sequence abolished photolabelling of TAF_{II}55 and p31, whereas crosslinking of TAF_{II}135 to this region was moderately enhanced (Fig. 1b, lanes 5 and 6). Furthermore, TFIIA strongly enhanced crosslinking of TAF_{II}135 at position -19 (Fig. 1c, lanes 11 and 12) and



moderately increased crosslinking of TAF_{II}95 at position -9/-8 (Fig. 1d, lanes 3 and 4). The most dramatic effects of TFIIA were evident near the transcription start site. As shown in Fig. 1d, TFIIA strongly induces crosslinking of TAF_{II}250 and enhances crosslinking of TAF_{II}135 at position -2, whereas crosslinking to TAF_{II}55, TAF_{II}46 and p31 is virtually abolished (compare lanes 7 and 8).

Binding of TFIIA may sterically hinder photolabeling of TAF_{II}55, p31 and TBP, but not of TAF_{II}135, upstream of the TATA box; however, the differential effects of TFIIA on the crosslinking of TAFs downstream of the TATA region indicate that TFIIA-mediated conformational changes occur within the TFIID nucleoprotein complex. They also suggest that TFIIA has a direct role in the relative positioning of promoter regions at and downstream of the transcription start site. This idea is supported by the observation that downstream interactions of TFIID on the Ad2ML promoter are slightly increased in the presence of TFIIA (Fig. 2, lanes 3 and 4) and are more resistant to challenge with excess carrier DNA (Fig. 2, lanes 8 and 9).

As TAF_{II}135 is crosslinked to promoter sequences immediately upstream and downstream of the TATA box (Fig. 1b, c), and also near and further downstream of the transcription start site (Fig. 1d, and data not shown), we considered that TFIID binding might position these promoter regions in relatively close proximity to each other. DNA wrapping by human TFIID was proposed earlier on the basis of a nucleosome-like DNase I protection pattern obtained when TFIID binds downstream of the Ad2ML TATA sequence⁵ (Fig. 2). Remarkably, recent biochemical²⁰ and crystallographic²¹ studies also demonstrate a histone-octamer-like organization of TAFs within TFIID. We therefore tested the effect of f:TFIID binding on the superhelicity of a circular DNA template. For this purpose f:TFIID was bound to radiolabelled, relaxed closed-circular plasmid DNA containing the Ad2ML promoter. After treatment with topoisomerase I, DNA-bound f:TFIID was removed by proteinase K treatment and phenol extraction. Analysis of the resulting topoisomers by two-dimensional agarose-gel electrophoresis revealed increased negative supercoiling of the DNA template (Fig. 3), suggesting that a left-handed superhelix formed upon f:TFIID binding. Nucleosome-like wrapping of DNA must be TAF-dependent because identical footprinting units of f:TBP had no effect, consistent with earlier results²².

On the basis of our results and earlier crystallographic data¹⁴⁻¹⁶, we propose a tentative topological model of the human TFIID-Ad2ML complex (Fig. 4).

e

	-46/-42		-31/-29		-30/-25		(-31/-29)/-19		-9/-8		-2	
TFIIA	-	+	-	+	-	+	-	+	-	+	-	+
TAF _{II} 250											+	++++
TAF _{II} 135	+	++					++	++++			+	++
TAF _{II} 95									++	+++	+	
TAF _{II} 55	++										+	
TAF _{II} 46											+	
TBP	+++											
TFIIA α	+	++++										
p 31	++++	+									+++	
TFIIA β			+	++++	+	++++	+	++++				

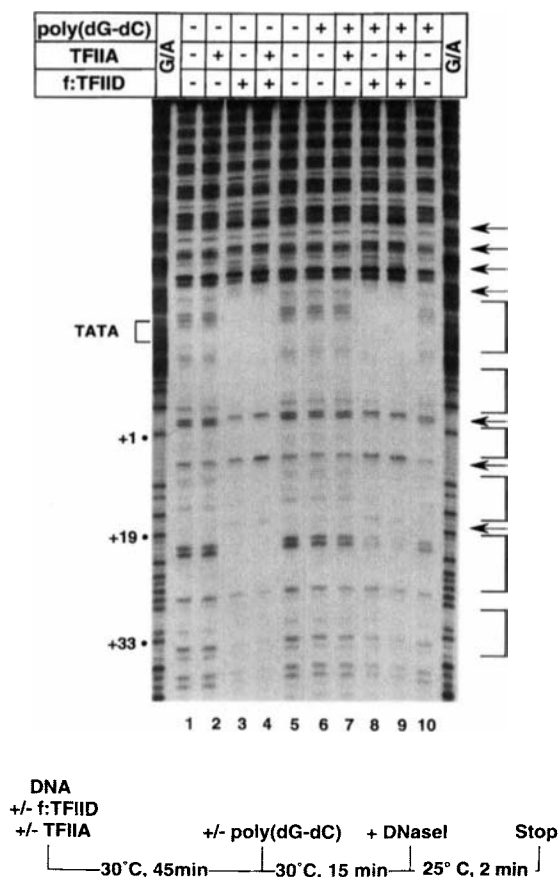
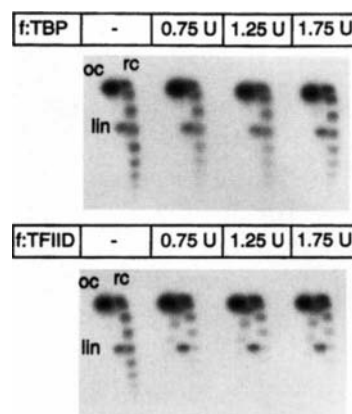


FIG. 2 TFIIA stabilizes human TFIID downstream interactions on the Ad2ML promoter, as shown by DNase I footprinting of TFIID complexes with the Ad2ML promoter formed in the presence or absence of TFIIA. Brackets indicate protected regions and arrows indicate sites of enhanced DNase I cleavage. Complexes were formed with 2 fmol 268-bp DNA template and two footprinting units of f:TFIID in 50 μ l 12 mM Tris-HCl, pH 7.9 (at 4 °C), 20 mM HEPES-KOH, pH 8.4, 5 mM MgCl₂, 60 mM KCl, 0.12 mM EDTA, 12% glycerol, 0.02 mg ml⁻¹ BSA (Boehringer Mannheim) and 10 μ g ml⁻¹ poly (dG-dC) (Pharmacia). Preformed complexes obtained after 45 min at 30 °C were further incubated for 15 min in the absence or presence of 100 ng poly(dG-dC). Specific DNA-binding activities of f:TBP or f:TFIID preparations were determined by DNase I footprinting assay; one footprinting unit corresponds to the amount of protein required for complete Ad2ML TATA-box protection in a 50- μ l binding reaction using 1 fmol 268-bp promoter probe.

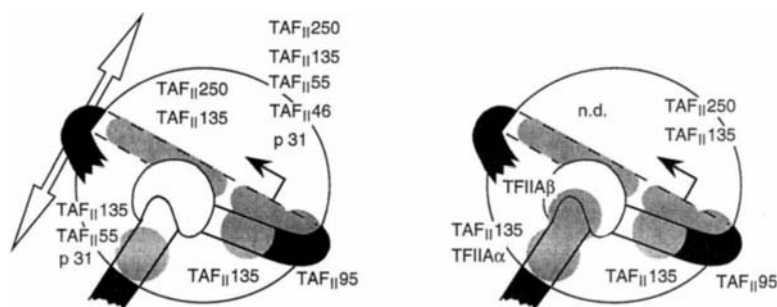
FIG. 3 TFIID binding to a circular DNA template containing the Ad2ML promoter leads to a change in superhelicity. DNA topoisomers were analysed by two-dimensional gel electrophoresis, with the first dimension run from top to bottom and the second run from left to right in the presence of ethidium bromide. Under these conditions, negatively supercoiled DNA migrates more slowly and positively supercoiled DNA migrates faster than relaxed circular DNA, respectively. Amounts of f:TBP or f:TFIID are indicated in footprinting units (as defined for Fig. 2). rc, Relaxed closed-circular DNA; nc, nicked circular DNA; lin, linear DNA. Note that binding to lower-affinity TATA sequences present in the plasmid DNA outside the Ad2ML promoter in the presence of saturating f:TBP or f:TFIID footprinting units contributes to the change in linking number by f:TFIID. On the basis of titration experiments using limiting amounts of f:TFIID, a linking number change of 1–2 can be attributed to Ad2ML promoter binding; under these conditions <30% of the template is bound and the empty control vector is unaffected (data not shown).

METHODS. Radiolabelled, relaxed closed-circular DNA was prepared as described^{28,29} using a unique *Bam*HI site in pTOG5ML (–51/+62). Complexes were formed at 30 °C (Fig. 2 legend) using 0.75 fmol radiolabelled relaxed circular pTOG5ML (–51/+62) (+). After 45 min, 5 units of topoisomerase I (Promega) were added, followed by further incubation for 15 min. Reactions were stopped by addition of 1 volume of 1% SDS, 20 mM EDTA, pH 8.0, followed by deproteinization with 10 µg proteinase K for



30 min at 37 °C. DNA was recovered by phenol treatment and ethanol precipitation and analysed by two-dimensional gel electrophoresis as described³⁰.

FIG. 4 A tentative model of the human TFIID–Ad2ML promoter complex. TAF interactions with promoter DNA downstream of the Ad2ML TATA box region, probably mediated largely by phosphate backbone interactions, result in the formation of a left-handed superhelical turn around the TFIID complex. Shaded areas indicate the relative positions of TAFs and TFIIA subunits to promoter-bound TBP and DNA. An arrow indicates the variable positioning of promoter regions downstream of the TATA-box region. Binding of TFIIA results in a conformational change within the TFIID nucleoprotein complex which leads to stable disposition of promoter DNA relative to TAFs. The path of the DNA can only be surmised, as the exact disposition of TAFs within TFIID is not known. However, a detailed DNase I footprinting analysis of the TFIID–Ad2ML promoter complex reveals that TFIID makes contact with mainly one face of a hypothetical B-form DNA extension downstream of the TATA box in the TBP/DNA co-crystal structure¹⁴, in favour of a model in which the promoter DNA downstream of the TATA box follows a left turn (T.O.,



TFIID-DNA

TFIIA-TFIID-DNA

unpublished observations), thereby positioning the transcription start site near the binding site for TFIIB^{23,24}.

Our model emphasizes a complex network of TAF–DNA contacts in the absence of a TFIID subunit homologous to *Drosophila* TAF_{II}150 (ref. 18) and predicts that TAF-dependent nucleosome-like DNA wrapping in the presence of TFIIA may position the transcription start site in the vicinity of the binding site for TFIIB (refs 23, 24). Thus, induction of TFIID downstream interactions

by transcriptional activators^{7–10}, in conjunction with TFIIA^{9,10} in a different core promoter context, might reflect the formation of a stereospecific TFIID nucleoprotein complex that permits efficient and functional recruitment of the remaining transcription factors. □

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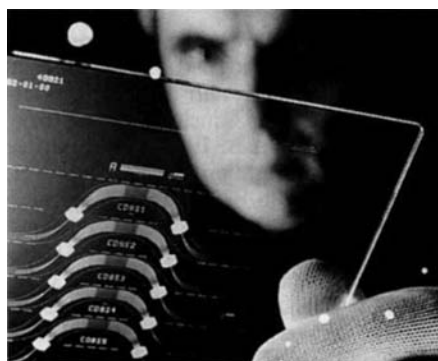
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A selection for the physical sciences

Today's special — product review offers problem solving software, information about nanotechnology funding, a diode-pumped solid-state laser and circuit simulation software.

A NEW device is available from Lucent Technologies that should significantly **improve the capacity of light-wave communications systems**. The 1450D dense wavelength division is used at the receiving end of a fibre-optic transmission link to sort and route light-wave signals carried on eight information channels, or wavelengths, within a single filament of optical



Light-wave communication systems.

fibre. In the broadband transmission system, which includes the 1450D DWDM, each channel carries 2.5 gigabits (billion bits) of information per second, making the total capacity 20 gigabits per second. At that rate, the system is able to transmit the equivalent of almost 5,000 novels in one second — about eight times as much as most long-distance fibre-optic systems. The 1450D DWDM, developed by Bell Labs Research and based on technology invented by Dragone, is fabricated using a planar-waveguide technology called silicon-optical bench

Reader Enquiry No. 100

System Dynamics International has introduced *e*, the **'evolutionary algorithm tool'** designed to solve very difficult problems in engineering and science. According to the manufacturer, the program uses evolutionary problem solving techniques modelled after biological processes, 'breeding' computer programs to solve complex problems. Starting initially with a random pool of computer programs, the system applies its principles to evolve progressively towards a fit solution. The main advantage of the program is that it is a complete stand-alone tool, which has the capability to output in C, Fortran or Basic. Designed to go beyond simple genetic algorithms, it encapsulates programs into reusable components and stores them in a library that

represents accumulated learning. Innovations include the fact that no prior assumptions are needed about the solution. It solves far more than just coefficients, giving entire sets of equations, whether or not the form is known in advance. An optional module converts the pseudocode into compilable C, Fortran or Basic source code. Solutions may then easily be incorporated into other programs.

Reader Enquiry No. 101

Safety eyewear

The new **laser safety spectacles** from Carl Zeiss are suitable for all uses in medicine and technology, offering good stability, temperature insensitivity and comfort. The type BG spectacles are made from grilamide with glass-fibre reinforcement and an aluminized surface. The plastic sides have a steel spring insert and are individually adjustable. For additional safety, an optional elastic head band is available, which fits into the side tips of the spectacles. Replaceable soft pads are recessed into the nose contact area, and lateral ven-



For Improved eye safety against technological hazards — see Carl Zeiss.

tilation areas with ray traps make them more comfortable to wear. The modern design of the type BG spectacles should ensure proper positioning of the filters with respect to the visual axes of the wearer, and provide a large, air-filled area behind the filters, which is surrounded on all sides by integrated, protective screens. The spectacles can be glazed with single-vision correction lenses. Protective filters for the entire radiation range of lasers are available.

Reader Enquiry No. 102

Spurred on by the latest European certification requirements, Glendale has redesigned its **laser safety eyewear range**. New sets designed for use with Ti:sapphire and diode lasers have been introduced; other less popular wavelength ranges have been dropped. A new full-view format has been added to the spectacle, goggle and wraparound styles already in existence. All models conform to the latest regulations. Glendale's polycarbonate construction results in a lightweight, comfortable and cost-effective design.

Reader Enquiry No. 103

Laser technology

Meridian Instruments has announced a new addition to their family of **confocal microscopes**. The Ultima-Z laser scanning confocal microscope offers high-quality imaging and scanning capabilities and is now available on a Zeiss Axiovert platform. The microscope features simultaneous ultraviolet and visible imaging, each under independent AOM control. With two exclusive scanning modes, the Ultima-Z brings both versatility and high resolution to confocal imaging. The StageScan mode permits imaging and quantification of large specimens, while the DualScan mode combines galvanometric mirror and stage scanning for rapid imaging of smaller areas. The Ultima-Z includes a comprehensive, user-friendly software package. Operation of the microscope is controlled with a mouse and pull-down menus. Complete three-colour and quantitative analysis is available for two-dimensional images, three-dimensional confocal reconstructions and independent or ratio kinetic data.

Reader Enquiry No. 104

Hamamatsu Photonics, manufacturers of high-quality electro-optic components, recently has developed a new series of **high-power pulsed laser diodes**. With a minimum radiant output of 20 W and high-speed response, these emitters are well suited to range finding and light-triggered control applications. As the emitting area is small these devices also provide good coupling to fibre-matched photodiodes. Moreover, amplifiers are available to give efficient conversion of light input to electrical output.

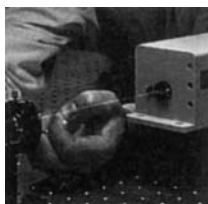
Reader Enquiry No. 105

A new 16-page, colour **catalogue** supplement from New Focus features a new Nirvana DC auto-balancing photoreceiver, fibre Bragg gratings from 3M, broadband isolators, and a new line of pint-sized mounts. The company is also introducing a new tunable diode laser with a centre wavelength of 980 nm, along with shorter lead times and higher output powers for its lasers. The Picomotor has a longer lifetime and an upgraded driver, featuring electronic switching and a GPIB. Of note, are the New Focus fibre Bragg gratings from stock and connectorized for convenience. The gratings should complement 3M's specialty optical fibres and provide passive wavelength filtering and stabilization in a compact all-fibre package. Applications include mirrors for fibre lasers, filters for WDM communication, as well as strain or temperature sensors.

Reader Enquiry No. 106

HMG Photonics has introduced the ICD-430 **diode-pumped, solid-state laser**. The new product uses the doubled output of a new Cr:LiSrAlF crystal that produces a 10-mW, single-mode output beam at 430 nm. This new laser should find use in data storage, bio-fluorescence and lithographic applications, being well suited to techniques requiring a high stability output of blue laser light. A high-speed feedback loop controls frequency instabilities and a second, lower frequency loop stabilizes the doubling crystal temperature to stay at peak power.

Reader Enquiry No. 107



New blue light laser.

Odds and ends

Titan software from Siemens can **simulate circuits with 1,000,000 transistors** long before an integrated circuit is produced in silicon, its behavior carefully tested using numeric simulation. Advances in the program now make it possible for circuits with more than 1,000,000 MOSFET transistors to be computed, providing a stated tenfold increase in power. By dividing the task among several processors, computing times can be achieved with the new software on a group of workstations comparable with those achieved using a supercomputer. The simulation of electrical circuits is a vital tool in the development of highly integrated components. However, the memory and performance requirements of simulation programs increasingly overload the capacity even of high-performance workstations. Researchers at Siemens have developed a method of 'area division' for circuit simulation whereby the circuit is broken down into partial circuits, which can be completed

The Foresight Institute, a non-profit organization dealing with nanotechnology-related issues, is offering a US\$250,000 (UK£160,000) **cash prize to the first individual or group to achieve specific major advances in molecular nanotechnology**. To win the newly announced Feynman grand prize, entrants must design and construct a functional nanometre-scale robotic arm with specified performance characteristics, as well as design and construct a functional nanometer-scale computing device capable of adding two 8-bit binary numbers. Nanotechnology is an emerging technology based on the ability to assemble individual molecules and atoms into precise structures. Its realization may allow the construction of supercomputers the size of a sugar cube, pollution-free manufacturing, as well as the development of molecular-scale robots that could repair damage in individual human cells (more than one billion such nanorobots would fit inside a single drop of blood).

Reader Enquiry No. 110

separately. A superordinate process is designed to ensure that the partial circuits concur at the 'edges', thus simulating the entire circuit.

Reader Enquiry No. 108

National Instruments has announced new SCXI systems for **remote or distributed data acquisition (DAQ)** applications. Remote communications are provided by two new products for SCXI, the SCXI-2000 chassis and SCXI-2400 modules, which add an RS-485/RS-232 interface to SCXI systems for long-distance communications that are compatible with the PC serial port. Both components include NI-DAQ driver software for Windows 95/3.1/3.1J and are compatible with LabVIEW, LabWindows/CVI and ComponentWorks instrumentation application software. Combining a wide variety of signal conditioning capabilities with a reliable long-distance communications link to the PC, an SCXI remote DAQ system delivers new flexibility to remote test and measurement applications.

Reader Enquiry No. 109

Kipp-Zonen has released a rugged and weather-resistant **net radiometer** that is designed to provide accurate and reliable measurements with four separate sensors of equal sensitivity. The CNR1 net radiometer, as it is called, should prove useful for the analysis of the solar and far-infrared radiation balance. Incorporating four individual sensors of equal sensitivity (two pyranometers for measuring the incoming and reflected solar radiation, and two pyrgeometers for measuring the incoming and outgoing far-infrared radiation), the CNR1 can measure parameters such as solar radiation, far-infrared radiation, albedo, energy balance and soil surface/sky temperature measurements, as well as net radiation measurement. Standard features include a built-in Pt-100 temperature sensor for accurate temperature measurement, an internal heating element for the elimination of frost



Net radiometer.

or dew deposition, a spirit level for convenient levelling, and a robust and weather-resistant housing requiring minimal maintenance.

Reader Enquiry No. 111

TA Instruments now offers a new **dynamic mechanical analyser (DMA 2980)** for evaluating the modulus and damping properties of materials, including polymers, ceramics and metals. It features multiple modes of deformation (single cantilever, dual cantilever, three-point bending, shear sandwich, compression and tension), frequency multiplexing (0.01–200 Hz), a broad temperature range (–150 °C to 600 °C), and a variety of sample forms (solids, viscous liquids, fibres and thin films) and sizes. The analyser can be connected to one of the company's family of thermal analyst controllers with OS/2-based thermal solutions software to form a multi-module thermal analysis system. The unit can also be connected to a rheology analyst controller and be run in combination with a controlled stress or controlled rate rheometer to provide full-range (liquid to solid) characterization of material deformation/flow properties.

Reader Enquiry No. 112

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